STRUCTURAL STUDIES ON VARICELLA ZOSTER VIRUS

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ABSTRACT

Title of Thesis: Structural Studies of the Varicella Zoster Virus

Jace Kellams Hougland, Doctor of Philosophy, 1990

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Little is known about the structure of varicella zoster virus (VZV) except what can be inferred from the more extensively studied herpes simplex virus (HSV). The core of VZV has only been visualized using thin sections of virions or infected cells. By using a unique technique called ion etching, which uses Ar^+ to strip away the outer layers of the virus, we have visualized what may be the intact core of the virion. We have seen toroidal structures approximately 70 nm in diameter, similar to those seen in thin section. We have also seen evidence suggesting that the toroids may be open-ended structures instead of closed rings.

To identify and further study VZV structural proteins we have cloned the following VZV open reading frames (ORFs) into the vaccinia shuttle vector pSC11: ORF11, ORF33, ORF34, ORF40, ORF47, and ORF66. These ORFs were chosen as potential structural genes based upon knowledge of their HSV homologues. Using these clones we constructed vaccinia recombinants for all but ORF11. Rabbits have been inoculated with these recombinants to create antibodies for use in determining if these are structural proteins, and for studies in cellular localization of the

proteins. The recombinant for ORF40, the major capsid protein, will also be used for studies on self assembly of the capsid.

Lastly, we have examined the protein kinase (PK) activity which is associated with purified VZ virions. Kinase activity was extracted from the virions and was fractionated using Amicon filters. When added to heat inactivated virions, the activities of the fractions suggested the presence of more than one PK in the virions. Autophosphorylation studies using purified virions revealed the presence of both cellular and viral PKs. To aid in identifying the viral PK we have cloned VZV genes 47 and 66, which contain eukaryotic PK domains, into the phagemid pBluescript® which allows *in vitro* expression of the genes. The major 175 kDa protein phosphorylated by the VZ virion kinases has been shown to be IE62, a phosphoprotein, whose HSV homologue is ICP4, a major regulator of the transcription of the viral genome.

STRUCTURAL STUDIES

OF THE

VARICELLA ZOSTER VIRUS

by

Jace Kellams Hougland

Dissertation submitted to the Faculty of the Department of Microbiology
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Health Sciences in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy 1990

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Lastly, I wish to thank my wife, Amy, for providing the support and understanding which allowed me to accomplish a project of this magnitude.

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INTRODUCTION

The *Herpesviridae* are a large family of viruses, which are widely disseminated throughout nature. Within this family, seven human herpesviruses have been identified (Roizman, 1990; Salahuddin *et al.*, 1986; Frenkel *et al.*, 1990), herpes simplex virus types 1 and 2 (HSV-1 and HSV-2), Epstein Barr virus (EBV), cytomegalovirus (CMV), varicella zoster virus (VZV), and the recently identified human herpesviruses 6 and 7 (HHV-6 and HHV-7). These all share common features such as an overall similarity in virion structure and the ability to form latent infections in their hosts, yet they give rise to considerably different clinical syndromes and possess quite variable genomic structures.

Varicella zoster virus is the cause of two specific clinical syndromes, varicella or chickenpox and zoster or shingles (Weller and Stoddard, 1952). The name "varicella" is derived from "variola" (for smallpox) and was a diminutive term ("little smallpox") while "zoster" is from the Greek "to gird", referring to the appearance of a dermatomal rash around the trunk. "Shingles" is derived from the equivalent latin verb "cingere" while "chickenpox" may be from the old English word "gican" - "itch" (Christie, 1974).

Varicella is normally a relatively trivial childhood rash which can spread rapidly and efficiently in appropriate circumstances, such as an elementary school classroom. It is also a potentially severe problem

for seronegative adults, leukemic children, and for infants who have acquired the disease *in utero* or immediately after birth. Varicella is characterized by a generalized rash which normally resolves without incident. The virus becomes latent in the sensory ganglia of the patient, and later, can be reactivated to cause zoster (Weller *et al.*, 1958). This disease is usually seen in older normal people and immunocompromised individuals (Hope-Simpson, 1965). It is usually confined to a single dermatome, and may leave neurological sequelae. Varicella can be acquired from a patient with zoster (the classic grandparent-to-grandchild spread) (Bruusgaard, 1932) but there is no evidence to support suggestions that zoster appears other than as a result of reactivation of the latent virus.

Throughout the history of infectious disease, there has been confusion between smallpox and chickenpox (not helped by some of the nomenclature discussed above). Now that smallpox has been eradicated (WHO, 1980), this problem no longer exists. Zoster, because of its unique appearance was, from earliest times, recognized as a specific clinical entity (Gordon, 1962), but it was not until 1888 that von Bokay (1909) actually recognized the link between varicella and zoster. Some years later, in a classic experiment, varicella was shown to result from inoculation of children with zoster lesion material (Bruusgaard, 1932). It was to be a further twenty years before the virus could be grown in cell culture (Weller and Stoddart, 1952) and even now, VZV remains one of the most difficult human viruses to work with, since its growth in cell culture is poor and cell-free virus is particularly unstable (Grose *et al.*, 1979). Nevertheless, much has been accomplished with VZV, especially in the last ten years, following the isolation of the viral genome and its

subsequent sequencing (Davison and Scott, 1986), and trials are being conducted of a vaccine (albeit not a perfect vaccine) (Takahashi, 1986) which may be licensed for restricted use in the United States before the end of this year (Gelb, 1990). In this context, an important goal for those in the VZV field remains the characterization of the infectious virus particle, its constituent proteins, and their role in the growth and spread of this ubiquitous human virus; such a study forms the basis for this dissertation.

The Virion

From the earliest days of electron microscopy, VZV was clearly distinguishable from smallpox virus, but similar to other herpesviruses (Ruska, 1943; Almeida *et al.*, 1962). The virion is 150-200 nm in diameter, with a lipid envelope containing viral glycoprotein spikes surrounding an approximately 100 nm icosadeltahedral (Caspar and Klug, 1962) nucleocapsid. Between these two structures lies the tegument, a poorly-defined proteinaceous layer of unknown function (Roizman and Furlong, 1974). Inside the capsid lies the genome, apparently organized in a "plug and toroid" nucleoprotein structure (Furlong *et al.*, 1972) (figure 1). The capsid consists of 162 capsomeres, with 12 pentons at the vertices and 150 hexons making up the 20 triangular faces (Wildy and Watson, 1963). It is characterized by having 5:3:2 axes of rotational symmetry (figure 2). In one of the more detailed early studies, which still has relevance to current work, Cook and Stevens (1970) found that virus released from human cells in tissue culture was often damaged or defective, with empty capsids, torn envelopes, peculiar morphology etc. In related work, Achong and Meurisse (1968) found that the particle:plaque

Figure 1. A drawing showing the components of the VZV virion. The virion consists of a DNA core surrounded by an icosadeltahedral nucleocapsid. The capsid is surrounded by an amorphous region of viral proteins called the tegument, which in turn is enveloped by a lipid membrane studded with viral glycoproteins.

VZV Virion

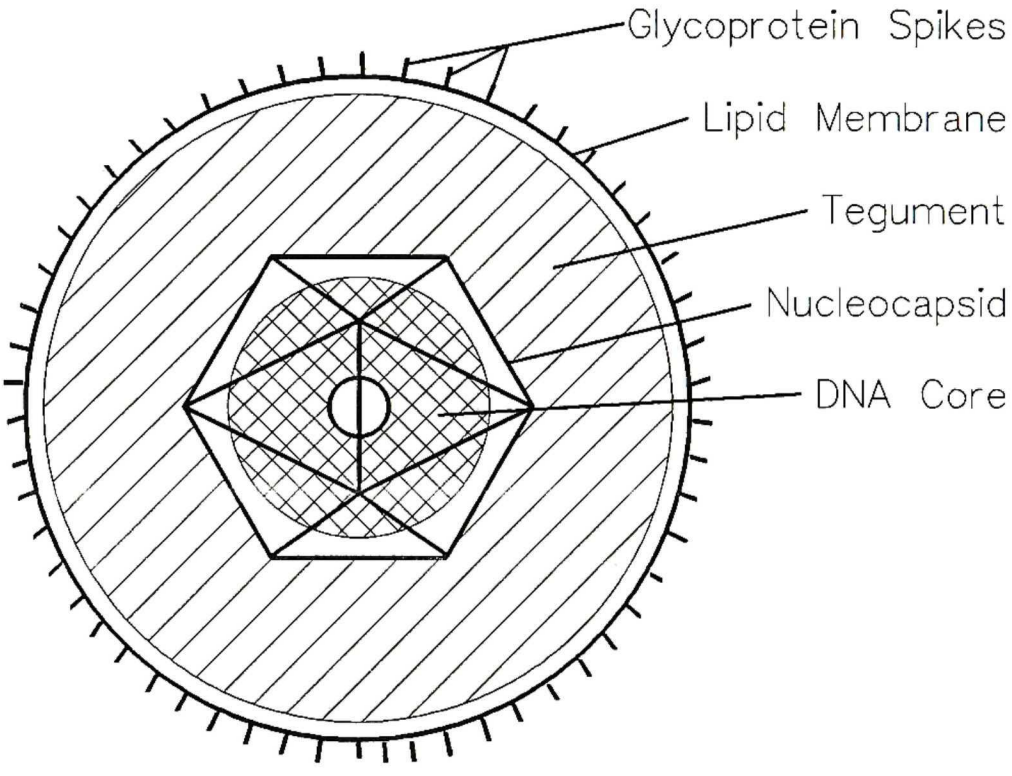
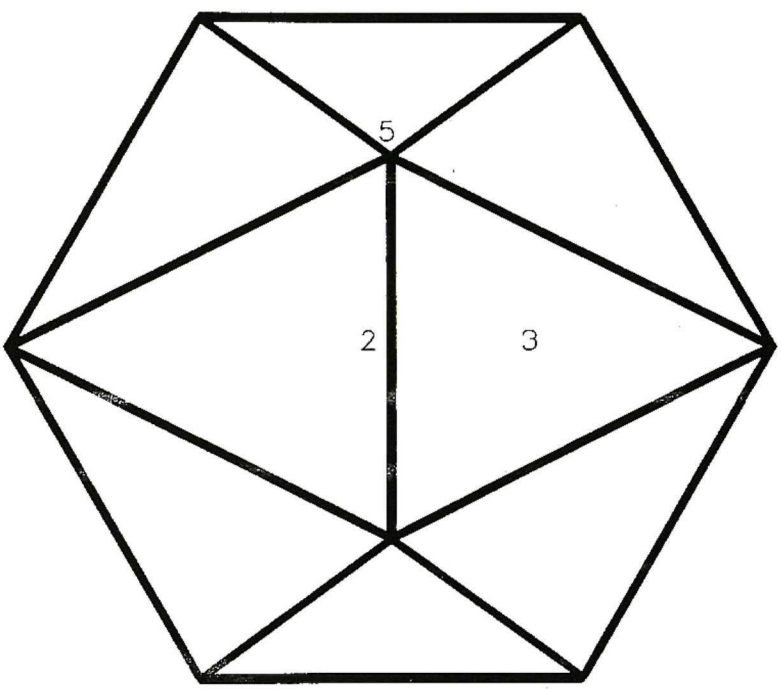

Glycoprotein Spikes
Lipid Membrane
Tegument
Nucleocapsid
DNA Core

Figure 2. A drawing of the VZV nucleocapsid. The capsid is an icosadeltahedron consisting of 162 capsomeres. The outer layer of the capsid is composed of 150 hexons which make up the 20 faces and 12 pentons which are found at the vertices. The 5:3:2 axes of icosahedral symmetry are indicated on the drawing.

VZV NUCLEOCAPSID



forming unit (pfu) ratio for VZV was about 10^6, several orders of magnitude greater than for HSV, for example. This bias towards damaged particles is one possible explanation for the poor yields of infectious VZV routinely obtained. An alternative explanation has been put forward by Gershon *et al.*, 1973, in which lysosomal enzymes are packaged within viral envelopes, resulting in degradation of viral structural components.

The VZV Genome

The characterization of the VZV genome effectively dates from the early 1980's, when two groups (Dumas *et al.*, 1980; Straus *et al.*, 1981) described the isolation of clean viral genomes using different methods of preparing virions and nucleocapsids, respectively. The genome is a linear dsDNA molecule of about 125,000 base pairs with a G+C content of 45% (Straus *et al.*, 1982). It is divided into a long (U_L) and a short (U_S) stretch of unique sequences bounded by, respectively, terminal and internal inverted repeat elements (TR_L/IR_L and TR_S/IR_S). The long unique element is about 105,000 base pairs (similar in size to several other human herpesvirus long unique elements), while the short unique element is only 5,200 base pairs. The short repeat elements are about 7,300 base pairs and their long counterparts are only 88.5 base pairs (Davison and Scott, 1986) (figure 3). The molecule exists in four isomeric forms, two of which predominate (Kinchington *et al.*, 1985), due to the inversion of the unique regions mediated by the inverted repeats. Since the U_S region inverts more readily than the U_L region, it is speculated that this disparity in the size of the repeat elements influences the extent to which the regions they bound are able to invert (Kinchington *et al.*, 1985).

Figure 3. A comparison of the structure of the genomes of HSV-1 and VZV. Both genomes have unique long and short regions (U_L and U_S) bounded by inverted repeats, the terminal repeats (TR_L and TR_S) and the internal repeats (IR_L and IR_S). The inverted repeats allow the unique regions to invert relative to each other, which in HSV DNA gives 4 equimolar fractions consisting of each possible orientation. The VZV unique long region rarely inverts, probably due to its relatively short terminal and internal repeats (not visible since drawing is to scale). Therefore VZV DNA has 2 approximately equimolar fractions due to the inversion of the unique short region.

HSV 1

VZV

Variation in restriction enzyme profiles among VZV isolates was detected early in the study of the viral genome (Straus *et al.*, 1983) and was subsequently shown to be the result of differences in the numbers of repeated sequence elements occurring in four regions of the genome (R1 through R4; Davison and Scott, 1986). These variable regions were used to study the epidemiology of VZV, showing among other things that varicella-derived virus was identical to zoster derived virus (Mishra *et al.*, 1984) and that varicella and zoster occurring in the same patient over a period of time were caused by the same virus (Straus *et al.*, 1984). There is also evidence from such studies that more than one VZV strain may exist in a patient at any one time (Hondo *et al.*, 1987). Interestingly, while there is considerable variation in the restriction enzyme profiles of DNA isolated from different individuals, a given strain is very stable to passage in cell culture (Hayakawa *et al.*, 1986).

The most important event in VZV molecular biology, however, occurred in 1986, when Davison and Scott (1986) published the entire nucleotide sequence of its genome. This study showed the DNA to vary in size among different isolates (a result of the repeat elements; see above), but on average to be about 124,884 base pairs and confirmed essentially all the data obtained earlier on the genome character. More importantly, however, it revealed that VZV could code for about 70 proteins, most of which were similar to those encoded by herpes simplex virus (HSV). Indeed, the nucleotide sequence data suggest that HSV and VZV are evolutionarily related and were probably derived from a common ancestor quite recently in time. There is also evidence that genes in the VZV U_L region are related to some of those in the Epstein Barr virus (EBV) genome

(Davison and Taylor, 1987) and that some homology exists between VZV and pseudorabies virus (PRV; Petrovskis *et al.*, 1986).

VZV Proteins

A by-product of the difficulty in growing VZV in cell culture has been that viral proteins remain poorly defined, and studies of polypeptides synthesized after VZV infection have been further complicated by a poor mechanism for the shut-off of host macromolecular synthesis existing in VZV. Nevertheless, information is available on certain viral proteins, notably those associated with the virion.

The first reports of effectively purified VZV particles were by Shemer *et al.* (1980) and Shiraki *et al.* (1982), who used a variety of techniques to isolate apparently host protein free VZV. Protein analyses of their products suggested that there were 30 to 35 structural proteins, including several glycoproteins and that the major protein (equivalent to the HSV VP5 - the major capsid protein) was about 145 kDa. A separate study (Asano and Takahashi, 1980) reported on polypeptides in VZV-infected cells and described over thirty of these; however, correlation of these data with the structural information from the other laboratories has proved difficult.

VZV proteins are produced in three different stages (Honess and Roizman, 1974). The first set of proteins produced are called "immediate early" (IE), which are made prior to the synthesis of any of the viral proteins and have to be made prior to the production of the "early" proteins. Production of the early proteins is required for the synthesis of the viral DNA, which must take place prior to production of the "late" proteins.

More recently, Shiraki and Hyman (1987) have reported that there are four immediate early proteins made after VZV infection and these, presumably, play a central role in the growth cycle of the virus. Among the putative early VZV proteins are a thymidine kinase (which also has deoxycytidine kinase activity) (Kit, 1979), a DNA polymerase activity (Mar *et al.*, 1978), and a major DNA-binding protein (Kinchington *et al.*, 1988). Among the late proteins, the VZV glycoproteins have received a great deal of attention.

There are five glycoproteins (Davison *et al.*, 1986) encoded by VZV, gpI, gpII, gpIII, gpIV, and gpV, equivalent to the HSV glycoproteins gE, gB, gH, gI, and gC, respectively. The most abundant of these is gpI, with gpIV representing the least abundant species. All VZV glycoproteins appear to be components of the virus particle, although it is not clear whether or not they are all necessary for infection. Each of the glycoproteins seems able to induce neutralizing antibodies against VZV, either in the presence or the absence of complement (Kinchington *et al.*, 1990). The current assumption is that the properties of VZV glycoproteins will parallel those of their HSV counterparts, but this may not necessarily be so. For example, it has not been possible to detect Fc-binding activity with gpI (Gelb *et al.*, 1981) as would be expected from its relation with HSV gE, and $C3_b$-binding activity has not been detected in VZV gpV, despite the ability of HSV gC to carry out this function (Ling, 1990).

Zweerink and Neff (1981) described seven nucleocapsid proteins, the major species among which was a 155 kDa polypeptide. More recently, Friedrichs and Grose (1986) have suggested that a nucleocapsid protein

(p32) may be involved in packaging viral DNA. Since there are seven VZV capsid proteins and five VZV glycoproteins then there may be as many as 20-25 structural proteins in the tegument, representing approximately 2/3 of the structural proteins of VZV.

With the generation of the VZV nucleotide sequence, it is now possible to identify open reading frames (ORFs) and attempt to match these with known proteins or with likely functions, by analogy with other known herpesvirus proteins. This approach has not yet yielded many new insights into VZV, although it has confirmed much of the data already published. One exception, however, is that an ORF encoding a protein with potential thymidylate synthetase activity has been detected through scanning of the VZV nucleotide sequence and the enzymatic function of the gene product has been confirmed using standard biochemistry (Thompson *et al.*, 1987). Interestingly, this is an activity possessed by only two herpesviruses, VZV and herpes virus saimiri (HVS), and may represent an important factor in differentiating the pathogenic characteristics of these two viruses from the other human herpesviruses.

VZV Transcription

In general terms, the transcription pattern of VZV mimics that of HSV, both in the location of transcripts for a particular gene and in their direction of synthesis (Ostrove *et al.*, 1985). All major transcripts appear to be polyadenylated, although two non-polyadenylated species of unknown function have been described (MacGuire and Hyman, 1986). Recently, the precise transcription pattern for specific VZV genes has begun to be investigated, and some differences from the HSV pattern and from "normal" eukaryotic systems are emerging. Davison and Scott

(1986) mapped transcripts for the VZV thymidine kinase gene, finding that there was a long untranslated leader sequence, perhaps contributing to the inefficiency of production of the thymidine kinase protein. McKee *et al.* (1990) investigated the mRNAs specific for the VZV IE62 ORF (the equivalent to the HSV ICP4) and found unusual TATA and polyadenylation signals, and Ling *et al.* (1990a and b) have examined both the gpIV and gpV mRNA patterns and also found unusual TATA motifs. What appears to be emerging from these data is that VZV does not seem to abide by the same rules as other herpesviruses, or other eukaryotic systems, for that matter, in its control of expression of its genome. Whether these unusual signals are directed towards some special purpose of the virus, or are trivial, remains to be seen.

The Structure of HSV

Of the currently-known human herpesviruses, HSV 1 has been the most extensively studied, particularly with respect to structure. Recently, its capsomeres have been shown to be arranged in a truly hexameric fashion, unlike those of any other known DNA virus (Steven *et al.*, 1986) and convincing computer-based models of its nucleocapsid structure have been devised (Schrag *et al.*, 1989).

The core and capsid of HSV 1 are made up of seven proteins of which VP5 (155kDa) is the most abundant (60%; Cohen *et al.*, 1980). Each hexameric capsomere is made up of six VP5 molecules, folded together to give a pear-shaped entity with a central hole which is larger at the top (outside) than at the bottom (inside). The composition of the pentameric capsomeres remains unresolved, but may also involve VP5. Other nucleocapsid proteins have not been assigned specific functions, but a 12 kDa

polypeptide has been suggested to have a function in DNA condensation (Preston *et al.*, 1983).

While VZV has five glycoproteins, HSV has eight, three of which have no obvious counterpart in the VZV-infected cell or in the virion. Not all of the HSV glycoproteins have been assigned specific functions, but the five with counterparts in VZV have Fc-receptor activity (gE and gI), cell fusion ability (gB), receptor recognition (gH), and $C3_b$-binding functions (gC). As discussed earlier, VZV glycoproteins do not necessarily share functions with their HSV homolog. One surprising comparison between HSV and VZV is that VZV has no equivalent to gD, the HSV glycoprotein which appears to be the most immunogenic and plays a major role in host cell recognition. It will be of interest to discover if VZV possesses an alternate protein or proteins with the same characteristics as gD.

Somewhat after the publication of the VZV nucleotide sequence (Davison and Scott, 1986), that of HSV1 was published; the delay was due to the difficulty in sequencing DNA with such a high proportion of G+C (McGeoch *et al.*, 1985; 1988). In many ways, this was a more useful piece of information than the VZV sequence, since so much more was already known about the growth cycle of HSV. From the perspective of this dissertation, it was possible, for example, to find the genetic loci for several HSV structural proteins (all the glycoproteins and several capsid proteins, including VP5). We were able to use these in assigning tentative map locations for analogous VZV proteins, then proceeding with identification accordingly. This aspect is discussed in detail in the results section.

Herpesvirus Expression

There is a carefully-controlled pattern of expression from herpesvirus genes after infection of a susceptible cell. Most of the data supporting this scheme has come from studies with HSV, but evidence from other systems suggests that it is generally applicable to all members of the family *herpetoviridae* (Honess and Roizman, 1974). The first event after successful penetration of the viral genome into the host cell nucleus is a wave of "immediate early" transcription, followed by translation of these messages. This leads to "early" RNA and protein synthesis, viral DNA synthesis and, finally "late" transcription and translation. All transcripts are produced through the host's RNA polymerase II (Honess and Roizman, 1974). The immediate early proteins function in controlling their own, as well as subsequent protein synthesis (Preston, 1979), the early proteins play roles in control of late transcription and in DNA replication (Conley *et al.*, 1981; Godowski and Knipe, 1983), while the late proteins seem largely to be structural proteins, with no obvious controlling or catalytic role. An exception to this is $V_{mw}65$, a structural polypeptide which is able to transactivate the gene for the major immediate early protein (ICP4); surprisingly, it has recently been shown that, although VZV possesses an analogous gene, its product, while structural, is unable to transactivate transcription from the IE genes (Everett, 1989).

VZV Latency

One of the most fascinating features of VZV infection is that the virus is able to disappear from the infected host soon after the initial acute (varicella) infection and remain dormant for, usually, sixty or more years, before reactivating to cause zoster. The mechanism of establish-

ment of the latent state in VZV infection is unknown but, in one of the more important papers in the field, Hope-Simpson (1965) has proposed a plausible pathway for reactivation of the virus. Essentially, his ideas revolve around deterioration of the host's immune system allowing active replication of VZV. Evidence in support of this hypothesis comes from observations that those with immunocompromised status are particularly susceptible to zoster (Juel-Jensen and MacCallum, 1972). While this general mechanism has much appeal, it leaves open questions such as whether VZV is reactivated on a frequent basis and is normally contained by the host or whether the release of virus at the ganglion is a very rare event.

Recently, there have been interesting developments in the molecular aspects of latency in HSV. Stevens *et al.* (1987) have proposed that a special HSV transcript (latency-associated transcript or LAT) is expressed only in the latent state and is the only HSV transcript present in latently infected neurons. It is nuclear and was originally thought, perhaps, to keep the virus dormant through an "antisense" mechanism, since the LAT was actually antisense to ICP0, a major HSV controlling protein. More recently, its function seems to be more plausibly associated with reactivation of HSV, in that mutants of HSV with no LAT are much less likely to reactivate from a rabbit ganglion than wild-type virus (Hill *et al.*, 1990).

The latent state of VZV was originally thought to be similar to that of HSV, but recent work suggests there are many differences (Croen *et al.*, 1987). Unlike HSV, which establishes latency in neurons, latent VZV is found in the neuronal satellite cells. Also, unlike HSV, transcription

from the VZV genome during latency involves a number of early functions; VZV has no LAT gene (Straus, 1990). These major differences between the two viruses may help to explain why VZV reactivates much less frequently than HSV (it has to pass from the satellite cells to the neuron and then to the nerve fibers). In addition, VZV causes much more extensive nerve damage than HSV does upon reactivation, and the pattern of latency/reactivation involving a productive infection of the satellite cells would be consistent with this observation.

The VZV Vaccine

At this moment, a live attenuated VZV vaccine (LAVV), the Oka vaccine strain of Takahashi (1986), is being considered for use in high risk populations in the USA, principally leukemic children. Studies both here and abroad indicate that the Oka vaccine is effective for more than 85% of recipients, with only mild complications (Gershon *et al.*, 1986). It has been proposed that this vaccine be added to the measles/mumps/rubella (MMR) vaccine currently given to children, and that this mixture become a routine treatment for all children (Arbeter *et al.*, 1986). However, there remain problems with the Oka vaccine which suggest that caution should be exercised before widespread use is recommended. Probably the chief among these is that the vaccine virus appears to be capable of establishing a latent infection which may reactivate and cause potential problems later in the life of the recipient (Hayakawa *et al.*, 1984), but there is also some possible difficulty in that the vaccine induces poor and/or waning immunity, leaving adults unprotected against wild-type VZV infection (Brunell, 1977).

Recently, investigations into the nature of the attenuation of

the Oka vaccine virus have indicated that it produces much smaller amounts of the glycoprotein gpV than does wild-type virus (Kinchington *et al.*, 1990) and that this glycoprotein is present in only very small quantities in the virus particle. Whether or not this lesion is the source of the vaccine's attenuated state, these data emphasize the importance of continuing studies to probe the structural features of VZV, and how they relate to the function of the virus.

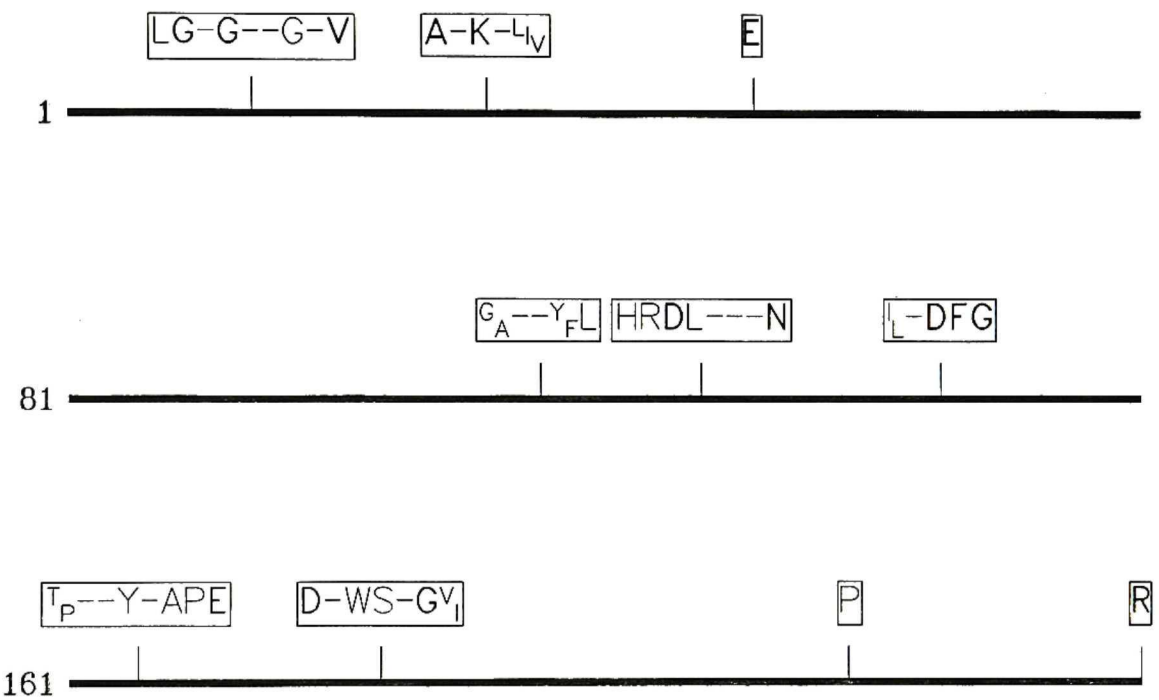
Viral Protein Kinases

Although cellular protein kinases have been studied for over thirty years (Krebs *et al.*, 1959), it was probably not until Collett and Erikson (1978) found that the *onc* gene of Rous sarcoma virus had such an activity that the majority of virologists became aware of the potential of these enzymes.

Protein kinases are enzymes which catalyze the transfer of phosphate from a nucleoside triphosphate (usually ATP; Hathaway and Trough, 1982) to the hydroxyl group of either serine/threonine or tyrosine, depending on the enzyme. Extensive analysis of amino acid sequences of eukaryotic protein kinases from a variety of sources reveals a kinase domain with considerable structural consistency (Hunter and Cooper, 1985). Figure 4 shows the most highly conserved motifs within the kinase domain. Interestingly, prokaryotic protein kinases posses a kinase domain bearing no resemblance to the eukaryotic domain (Cortay *et al.*, 1988).

Many retrovirus *onc* gene products are cellular protein kinases accidentally acquired from the host cell (usually tyrosine kinases; Sefton, 1985) and the T antigens of papovaviruses appear to be associated

Figure 4. A map of the eukaryotic protein kinase domain showing the major motifs found within the domain (based upon: Leader and Katan, 1988). Those amino acids in bold are usually invariable, while the rest show the amino acid(s) which appear most frequently at these positions.



Eukaryotic Protein Kinase Domain

in the plasma membrane with cellular protein kinases which may be important for their cellular transforming action (Schaffhausen *et al.*, 1982). Aside from these retroviral protein kinases, the only three viruses known to encode such an enzyme are bacteriophage T7, vaccinia, and several of the alphaherpesviruses.

Interestingly, several viruses, such as vesicular stomatitis virus (VSV) and adenovirus appear to incorporate a protein kinase in their virions, but these enzymes are of cellular origin (Clinton *et al.*, 1982; Akusjarvi *et al.*, 1978). HSV virions also contain protein kinase activity, consisting of both cellular casein kinase II and a yet unidentified viral kinase (Stevely *et al.*, 1985).

The T7 phage-encoded protein kinase is a serine kinase which probably phosphorylates a protein (possibly the B' subunit of RNA polymerase?) which causes inhibition of host RNA synthesis; mutants in the phage kinase do not shut off host or early phage transcription (Ponta *et al.*, 1974). The gene (U_s3) encoding HSV protein kinase is found in the genomes of all alphaherpesviruses and was discovered as a result of sequencing the HSV genome (McGeoch and Davison, 1986). Extracts of HSV-infected cells contain a protein kinase activity with characteristics unlike any known cellular kinase. This activity could not be detected when deletion mutants in U_s3 were used to infect cells (Purves *et al.*, 1987). As far as can be discerned, these deletion mutants grew as well as wild-type virus in cell culture but the influence of the viral protein kinase on the growth of HSV in animal models remains to be tested.

As important as the protein kinase itself is the protein or proteins which it phosphorylates, since these presumably are the

functional molecules through which the regulatory action of the kinase is achieved. An excellent example of this is the role that protein kinases play in the regulation of the cellular division cycle in yeast. One control point is regulated by a cascade of at least three protein kinases (Reed *et al.*, 1985; Simanis and Nurse, 1986; Russell and Nurse, 1987a&b). Some kinases will autophosphorylate, but it is not known whether this has any functional significance. In the case of HSV, a number of potentially important viral proteins have been shown to be substrates for protein kinases, including the immediate early proteins ICP0, ICP4, ICP22 and ICP 27, and the alpha trans-inducing factor, $V_{mw}65$ (Marsden *et al.*, 1978; Ackerman *et al.*, 1984). Since these proteins all play roles in the control of HSV gene expression, it is reasonable to speculate that phosphorylation of them may modulate their action and, through it, the growth cycle of the virus.

Aims of the Present Work

One of the most important features of a virus is its structure and the macromolecules from which it is constructed. While the DNA of VZV particles has been the focus of a number of in-depth studies, virion proteins have been much less carefully examined and the actual structure of the VZV particle remains essentially unexplored. Given this lack of knowledge of the structure of VZV or of its proteins, it was decided to attack these problems using a number of approaches. The first was to use "ion etching" in an attempt to expose the internal features of the VZ virion for viewing with an electron microscope, with particular emphasis on the core structure. Then, we would attempt to map on the VZV genome the genes for a number of VZV structural proteins, including the major

capsid protein. In the course of this work, we hoped to be able to generate vector systems which express these viral proteins, allowing further work to proceed on their synthesis, cellular location, involvement in the immune response to VZV and ultimately, perhaps, on their role in the structure or assembly of the VZV particle. Finally, focussing on structural elements in the VZV virion which may have additional functions, we have explored the nature of protein kinase activities associated with VZV particles and have identified and expressed two putative protein kinases encoded in the VZV genome.

II

MATERIALS AND METHODS

Virus Propagation

Cells

All cells for tissue culture were grown in Eagle's Minimal Essential Medium with gentamicin (100 μg/ml) and sodium bicarbonate (0.2% (w/v)) (MEM). The medium used to grow the human foreskin fibroblast cell line (HFF) was supplemented with fetal bovine serum (FBS) at a concentration of 5% (v/v) and Serum Plus® (Hazelton Research Products, Inc., Lenexa, KS) (SP) at a concentration of 5% (v/v). All other cells were grown in MEM with 10% (v/v) SP. Cells were grown at 37°C in an atmosphere containing approximately 7% CO_2.

Varicella Zoster Virus

Varicella zoster virus (VZV) strain Scott, a clinical isolate, was kindly provided by Dr. G. Fischer, Dept. of Pediatrics, USUHS. It was grown in HFF cell strain USUHS 184, also generously donated by Dr. Fischer. VZV-infected cells were maintained by splitting a confluent 150 cm^2 flask of HFF cells and 1 day later adding 1/10 of a 150 cm^2 flask of VZV infected HFF cells, showing 50-80% cytopathic effect (cpe).

Stock VZV was made in the following manner. Infected 150 cm^2 flasks of cells showing 50-80% cpe were rinsed with phosphate buffered saline (PBS) and overlaid with 3 ml/flask of 0.25% (w/v) trypsin (Hazelton

Research Products, Inc., Lenexa, KS). After the cells detached, 7 ml/flask of MEM-5%FBS/5%SP were added and the cells were pelleted by centrifugation in an IEC (model HN-SII) benchtop centrifuge at 1,500 rpm for 5 min. The pelleted cells were resuspended in 1 ml/flask of freezing medium (MEM with 10% [v/v] FBS and 10%[v/v] dimethylsulfoxide [DMSO]), aliquoted into 0.5 ml aliquots, frozen stepwise (1 hr. each at 0°C, -20°C, and -80°C), and stored in liquid nitrogen.

For viral purification, VZV was either grown in 750 cm^2 roller bottles or 150 cm^2flasks. Each roller bottle was set up using 2½ confluent 150 cm^2 flasks of uninfected cells and ½ 150 cm^2 flask of infected cells, at approximately 80% cpe, per roller bottle. Flasks were set up as described above for virus maintenance.

Herpes Simplex Virus

Herpes simplex virus type 1 (HSV-1) strain F was kindly provided by Dr. Frank Jenkins, Dept. of Microbiology, USUHS. HSV-1 was grown in the Vero (African Green Monkey kidney, ATCC) cell line.

Stock HSV was made by infecting 150 cm^2 flasks of confluent cells with cell-free virus at a multiplicity of infection (moi) of 0.01 pfu/cell. Cells were overlaid with 5 ml/flask of MEM containing the virus and the virus was allowed to adsorb for 1 hr. at room temperature with rocking. Twenty ml/flask of MEM-10%SP was added and the cells were incubated at 37°C until a cpe of 80-100% was reached. The infected cells were trypsinized, pelleted, and resuspended in 1 ml of MEM-10%SP per 150 cm^2 flask. The infected cells were then sonicated in a bath sonicator (Heat Systems Ultrasonics, Inc., Plainview, L.I., NY), and quick frozen in dry ice and ethanol. Stocks were stored in liquid nitrogen.

Vaccinia Virus

Vaccinia virus strain WR (generously donated by Dr. B. Moss, NIAID, NIH, Bethesda, MD) and its recombinants were grown in CV-1 (African Green Monkey kidney) cells in MEM-10%SP. Stock virus was prepared and stored in the same manner as described above for HSV.

Thymidine kinase minus (TK^-) vaccinia recombinants were grown in human TK^- 143 cells (Rhim *et al.*, 1975; also a generous gift from Dr. B. Moss) in MEM-10%SP. Occasionally, the 143 cells were passed with 20 μg/ml 5-bromodeoxyuridine (BUdR; Sigma Chemical Co., St. Louis, MO) added to the media to screen out TK revertants.

Plaque Assay

Both HSV and vaccinia were titrated using the plaque assay method of Dulbecco (1952). Serial dilutions of virus were made in MEM-5%FBS/5%SP and 0.5 ml aliquots were put, in duplicate, onto confluent CV1 cells in six well tissue culture plates. The virus was allowed to adsorb for 1 hr. at room temperature with rocking. Unadsorbed virus and media were then aspirated and the cells were overlaid with MEM with 0.5% (w/v) methylcellulose, and incubated for 48 hr. at 37°C. The cells were then stained with 0.1% (w/v) crystal violet in 50% (v/v) methanol for 10 min., the excess stain was aspirated, and the cell sheet was allowed to air-dry. Plaques were counted and viral titers determined.

Polyacrylamide Gel Electrophoresis

Proteins were separated using one dimensional denaturing sodium dodecyl sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) (Laemmli, 1970). The gels were run on Bio-Rad Protean® II or Mini-Protean® II

vertical gel rigs (Bio-Rad Laboratories, Rockville Centre, NY).

Gels were made from a stock acrylamide solution containing 29.2% (w/v) acrylamide and 0.8% (w/v) N,N'-methylene-bisacrylamide (bis). Upper gel buffer (UGB) consisted of 125 mM Tris (pH 6.8) and 0.1% (w/v) SDS, and lower gel buffer (LGB) consisted of 375 mM Tris (pH 8.8) and 0.1% (w/v) SDS. A stock solution of 10% (w/v) ammonium persulfate (APS) was also made, which along with N,N,N',N'-tetramethylethylenediamine (TEMED) is required for the cross-linking of the acrylamide. Running buffer consisted of 25 mM Tris base, 192 mM glycine, and 3.5 mM SDS.

A 2-3 mm plug was poured at the bottom of each gel, to prevent leaking, consisting of 22.5% (w/v) acrylamide in LGB with 7.5 μl/ml APS and 7.5 μl/ml TEMED. The separating gel was usually 10% (w/v) acrylamide in LGB with 3.75 μl/ml APS and 0.75 μl/ml TEMED, but varied depending upon the size of the protein(s) of interest (e.g., 15% gels for proteins with a MW below 20 kDa). The stacking gel consisted of 4.5% (w/v) acrylamide in UGB with 5 μl/ml APS and 2.5 μl/ml TEMED.

Samples, in sample buffer (2% [w/v] SDS, 4% [v/v] glycerol, 720 mM β-mercaptoethanol, 50 mM Tris [pH 6.8], 400 μg/ml bromphenol blue, and 400 μg/ml phenol red), were heated in a boiling water bath for 5-10 min. before loading onto the gel. Mini-gels were run at a constant 200 V until the phenol red entered the plug (usually about 30 min.). Full sized gels were run at 15 mA/gel until the dye front entered the separating gel, and then at 30 mA/gel until the phenol red ran into the plug. Large gels were run using a water-cooling apparatus while mini-gels were run without cooling.

Western Blotting

Proteins separated by SDS-PAGE were electroblotted to either 0.45 μm pore size nitrocellulose (Schleicher & Schuell, Keene, NH) or 0.45 μm pore size Immobilon® P (polyvinylidene difluoride [PVDF], Millipore Corp., Bedford, MA) using Bio-Rad Trans-Blot® or Mini-Trans-Blot® rigs (Towbin *et al.*, 1979).

After electrophoresis, gels were soaked for 30 min. in WB-1 (219 mM glycine, 25 mM Tris base, and 20% [v/v] methanol) to get rid of the salts from the electrophoresis buffer. The gels were sandwiched, next to wetted nitrocellulose or Immobilon, between Whatman 3MM filter paper and Scotch-brite pads in accordance with the Bio-Rad instructions for the respective rigs. Mini-blots were run for 1 hr. at 100 V and large blots were done overnight at 300 mA/blot with cooling.

Following blotting, the blots were blocked by soaking them in WB-2 (7.5 mM Tris base, 3 mM Na-Azide, 150 mM NaCl, 42 mM Tris HCl, 4 mM EDTA, and 0.05% [v/v] Tween 20) with 10% (w/v) non-fat dry milk for 1 hr. at room temperature. Blots could then be stored at 4°C in WB-2 with 1% (w/v) milk, or probed with antibody.

To probe, blots were sealed in plastic bags with antibody diluted in WB-2 with 1% (w/v) milk and incubated at room temperature for 1-3 hr. with rocking. The blots were then washed 3 times, for 5 min. each, in WB-2 and labelled by sealing them in plastic bags in WB-2 with 1% (w/v) milk and 1 μl/ml ^{125}I labelled staph A (127 nCi/μl, New England Nuclear, Wilmington, DE [NEN]). They were incubated at room temperature for 1 hr. with rocking. Following labelling, the blots were washed 5 times, for 5 min. each, in WB-2 and put to film for autoradiography.

Agarose Gel Electrophoresis

DNA fragments were separated using agarose gel electrophoresis (Maniatis *et al.*, 1982). Gels were usually 1% (w/v) agarose in TBE (89 mM Tris base, 89 mM boric acid, and 2 mM EDTA), but the concentration of agarose was varied depending upon the size of the DNA fragments being looked at. A 10X sample buffer was used consisting of 0.25% (w/v) bromphenol blue, 0.25% (w/v) xylene cyanol, and 25% (w/v) ficoll. Following electrophoresis, the gels were stained with ethidium bromide (2 gtt of 5 mg/ml in 2-3 l of water) and the DNA was visualized using UV light. If DNA fragments were to be eluted from the gel for cloning, long wave UV light was used to visualize the DNA to minimize damage to the DNA.

Southern Blotting

DNA fragments separated by agarose-gel electrophoresis were blotted to Biodyne® A nylon membranes (Pall Ultrafine Filtration Corp., Glen Cove, NY) (Southern, 1975). The buffers used for southern blotting were those recommended by the manufacturer.

Following electrophoresis the agarose gel was denatured in 1.5 M NaCl, 0.5 M NaOH for 30 min. at room temperature with rocking. The gel was then neutralized in 3 M Na-acetate (pH 5.5) for 30 min. at room temperature with rocking. The gel was then placed on a sheet of filter paper, with its ends in 20X SSC (3 M NaCl, 0.3 M Na-citrate [pH 7]), and a sheet of Biodyne®, 2 pieces of thick filter paper, and a 3 in. stack of paper towels were placed on top of it. A weight was placed on top of this stack and the SSC was allowed to absorb up through the gel and into the paper towels for overnight. The membrane was then baked at 80°C for 1 hr.

Colony blots were carried out in a similar manner. Bacteria were either grown on Biodyne circles on the surface of bacterial plates or the

bacterial colonies were allowed to adhere to Biodyne by placing a disk of it onto a bacterial plate containing colonies of interest. The Biodyne was then denatured (as above) for 5 min., renatured (as above) for 5 min., and air dried for 30 min. It was then baked for 1 hr. at 80°C.

Following blotting the DNA was probed with DNA that had been labelled by either random primer labelling (Feinberg and Vogelstein, 1983) or using polynucleotide kinase (Chaconas and van de Sande, 1980). Synthetic oligonucleotides were kinase-labelled, while longer fragments were random primer-labelled.

The blots were prehybridized by being sealed in a plastic bag with hybridization solution (5X Denhardt's buffer, 5X SSPE, 0.2% [w/v] SDS, and 500 μg/ml denatured salmon sperm DNA) for 1 hr. at 68°C. Following prehybridization, denatured probe was added, the bag was resealed, and the blot was incubated for several hr. at 68°C. Following hybridization the blot was washed 3 times in wash buffer (5 mM Na-Phosphate [pH 7], 1 mM EDTA, and 0.2% [w/v] SDS), for 30 min. each, at room temperature with rocking. Following washing the blots were put onto Kodak XAR-5 film.

Virion Purification

HSV and VZV

Both HSV and VZV were purified in the same manner, using, as a basis, the method described by Roberts (1984), with modifications to optimize the technique. Prior to homogenization, for example, infected cells were suspended in TE buffer instead of PBS. Since TE is hypotonic, the cells readily swell when suspended in it, making it easier to disrupt

their plasma membrane during homogenization, allowing more gentle mechanical shear. The buffer used to make the gradients was also changed to a higher concentration of NaCl and a small amount of EDTA added, to decrease the possibility of extraneous proteins adhering to the virions. A higher concentration of sucrose was used in the sucrose gradient which was effective in holding back more of the contaminating material. Finally, the concentration of potassium tartrate was lowered in the glycerol/KT gradient which gave better separation between the virion band and the band above it. Also, at the higher concentration, the KT would occasionally crystallize during centrifugation; this could lead to disruption of the gradient as well as possible rotor failure.

Infected cells showing 80-100% cpe were harvested by scraping the cells into the media. The cells were pelleted by spinning for 15 min. in a GSA rotor at 3000 rpm. The cell pellet was washed by being resuspended in 10 ml of phosphate buffered saline (PBS) per 750 cm^2 of infected cell layer, and then pelleted again by centrifugation for 10 min. at 1,500 rpm in the benchtop centrifuge.

The cell pellet was resuspended in 1 ml of TE buffer (10 mM Tris [pH 7.4], 1 mM EDTA) per 750 cm^2 and homogenized for twenty strokes in a glass homogenizer with a motor-driven Teflon® pestle. The nuclei were then pelleted by spinning for 10 min. in the benchtop centrifuge at 2,500 rpm, and the supernatant, containing the virions, was collected.

The supernatant was layered onto a continuous 20-50% sucrose (w/v in 350 mM NaCl, 1 mM EDTA, 10 mM Na_2HPO_4 [pH 7.4] [gradient buffer]) gradient which was spun for 1 hr. at 17,000 rpm in an SW-28 rotor. When illuminated from the bottom the virions appeared as a diffuse cloud oc-

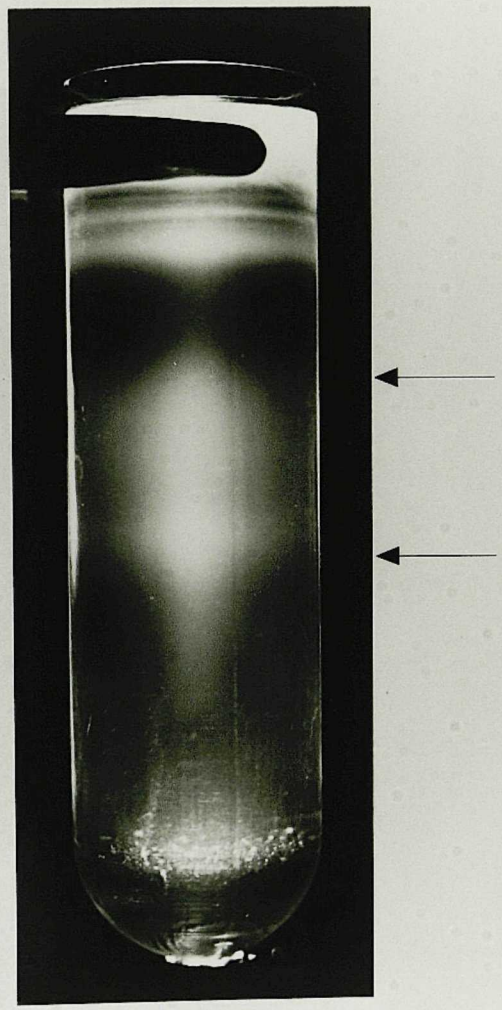
cupying the central portion of the tube (figure 5). Above and below the virions were bands containing cellular and viral debris. The material above the virions was removed by aspiration, and the virion band was collected. The virions were diluted with PBS and pelleted by spinning for 1 hr. at 25,000 rpm in an SW-28 rotor. The virion pellet was overlaid with 1 ml of PBS and put at 4C overnight.

The virion pellet was sonicated in the bath sonicator until the pellet dispersed. The virions were then layered onto a continuous 40% glycerol (v/v in gradient buffer) 40% sodium potassium tartrate (w/w in gradient buffer) (glycerol/KT) positive density/negative viscosity gradient (Obijeski *et al.*, 1974) and spun for 17 hours at 25,000 rpm in an SW-28.1 rotor. Illumination from the bottom revealed two bands in the bottom half of the tube (figure 6). The top band contained cellular debris, nucleocapsids, and some virions which appeared to be damaged. The debris was removed by aspiration. The second band, which had a clumpy appearance, contained mostly intact virions. The virions were collected, diluted with PBS, and pelleted for 1 hr. at 25000 rpm in an SW-28.1 rotor. The pellet was overlaid with 1 ml of PBS and put at 4C for overnight. The pellet was dispersed using the bath sonicator. The glycerol/KT gradient could be repeated to further purify the virions if necessary. The amount of protein present in purified virions was determined by the method of Bradford (1976).

Vaccinia Virus

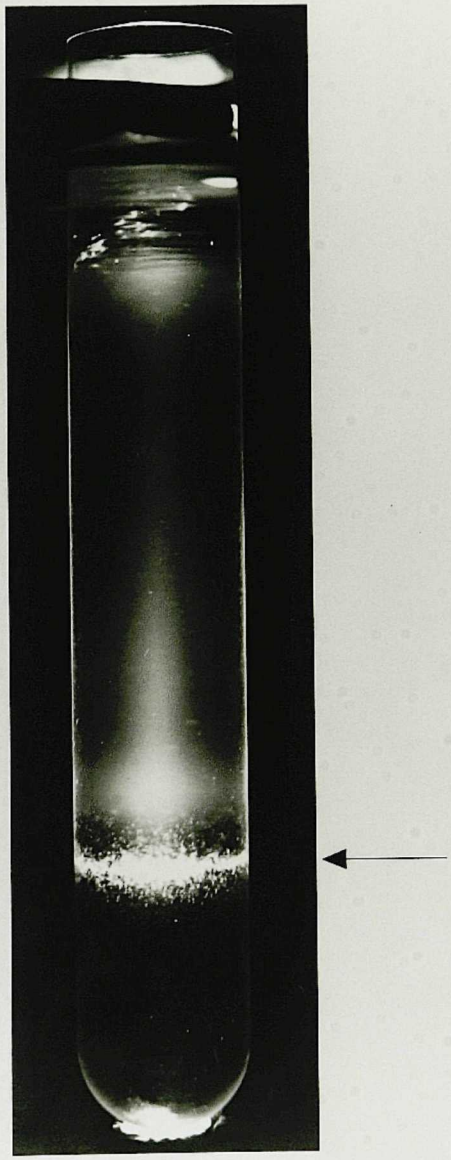
Vaccinia virus and its recombinants were purified using the following technique. Infected cells, showing 80-100% cpe, were scraped into the media and then pelleted for 15 min. at 3000 rpm in a GSA rotor.

Figure 5. A picture of a 20-50% (w/v in gradient buffer) sucrose rate/zonal gradient used in the preparation of VZV virions. A cytoplasmic extract from VZV infected cells was layered onto the linear sucrose gradient and was spun for 45 min. at 17,000 rpm in an SW-28 rotor at 4°C. When illuminated from below a diffuse cloudy region (between the arrows) was revealed which contained the virions.



Sucrose Rate/Zonal Gradient

Figure 6. A photograph of a 40% (v/v in gradient buffer) glycerol 40% (w/w in gradient buffer) potassium tartrate (KT) positive density, negative viscosity gradient. Partially purified virions from a sucrose rate/zonal gradient were layered onto the gradient and were spun for 17 hr. at 25,000 rpm in an SW-28.1 rotor at 4°C. When illuminated from below, the virions appeared as a clumpy band near the bottom of the tube (arrow). The band above the virions contains cellular membranes, disrupted virions, nucleocapsids, and other debris.



Glycerol/KT Density Gradient

The cells were then washed by being resuspended in approximately 2.5 ml of PBS per 750 cm^2 of cells and centrifuged for 10 min. at 1500 rpm in the benchtop centrifuge. The cells were resuspended with enough TE to make a final volume of approximately 2.5 ml per 750 cm^2 of cells, sonicated with a probe sonicator (Heat Systems Ultrasonics, Inc., Lenexa, KS) for 10 sec. at a setting of 5, layered onto a continuous 20-50% (w/v in gradient buffer) sucrose gradient, and centrifuged for 1 hr at 17,000 rpm in an SW-28 rotor. A moderately diffuse band containing the virus was visible in the top half of the tube when it was illuminated from below. Above the virion band was cellular debris which was aspirated prior to collecting the virions. The virions were diluted with PBS and then pelleted at 25,000 rpm in an SW-28.1 rotor for 1 hr. The viral pellet was overlaid with 1 ml of PBS and resuspended by sonication in the waterbath sonicator. The vaccinia virus was then quantitated by plaque assay.

Purity of VZV Virions

^{35}S-Methionine Labelling

The proteins from a 150 cm^2 flask of HFF cells were labelled with ^{35}S-methionine (NEN) by growing the cells for 48 hr. at 37°C in methionine-free MEM with 1/5 volume of regular MEM, 10% (v/v) FBS, and 15 μCi/ml ^{35}S-methionine. The labelled cells were scraped into the medium, pelleted, and resuspended in 5 ml of PBS. They were added to VZV infected HFF cells from 8 roller bottles that also had been scraped, pelleted, and resuspended in PBS. The cells were then pelleted and the cell pellet was processed, as described above, for virions. Samples from the following purification steps were collected for protein determination and radio-

activity measurement: homogenized cells, the cytoplasmic fraction, the pelleted band from the sucrose gradient, the pelleted band from the first glycerol/KT gradient, and the pelleted band from the second glycerol/KT gradient.

Following protein determination, 1 μg and 10 μg samples of each fraction were spotted onto 2.4 cm Whatman 540 filter paper circles and TCA (trichloroacetic acid) precipitated by dropping the circles into ice cold 5% (w/v) TCA containing 10 mM sodium pyrophosphate. After 10 min. the TCA was poured off and fresh ice cold TCA was added. After another 10 min. the TCA was replaced with ice cold 1 N HCl. After 10 min. the disks were put into ice cold 95% (v/v) ethanol for 5 min. The disks were then dried under a heating lamp, immersed in 5 ml/disk of Betafluor® (National Diagnostics, Manville, NJ), and the counts per minute (cpm) were measured on a Beckman LS9000 scintillation counter. The cpm/μg of protein and the percentage relative to the cellular homogenate were then calculated for each fraction.

Electron Microscopy

Purified virions were routinely examined in the electron microscope to evaluate their homogeneity. Virions were prepared for viewing by laying a formvar/carbon-coated copper grid onto a 15 μl drop of the virions for 1 min. The excess liquid was wicked off the grid, using bibulous paper, and the virions were negatively stained (Brenner and Horne, 1959) by laying the grid on a drop of 2% (w/v) phosphotungstic acid (PTA) in 10 mM Tris (pH 7.5) for 1 min. Excess liquid was wicked off and the grid was allowed to air dry for 5 min. Grids were viewed with a JEOL 100cx transmission electron microscope (JEOL USA Inc., Peabody, MA) oper-

ated at 80 kV.

Polyacrylamide-gel Electrophoresis

Virion purity was also looked at using SDS-PAGE. A 4-5 μg sample was electrophoresed along with an equivalent amount of uninfected and infected cells. The infected and uninfected cells were made by resuspending a PBS washed cell pellet, from 1 150 cm^2 flask, with 250 μl of 4X sample buffer and enough PBS to give 1 ml final volume. Following electrophoresis, the gels were stained using the colloidal Coomassie Brilliant Blue (CBB) G-250 technique described by Neuhoff *et al.* (1988). The virions would be compared with the uninfected and infected cell lanes to see if cellular proteins were present.

Ion Etching

Ion etching (Newcomb *et al.*, 1984) was done in the laboratory of Dr. Jay C. Brown at the University of Virginia School of Medicine, Charlottesville, VA.

Sample Preparation

Purified VZV was allowed to adsorb to formvar/carbon-coated copper grids by laying the grids on 10-15 μl drops of the virus for 1-2 min. The grids were transferred to a drop of water for a few seconds and then transferred to a 1.5 ml Eppendorf tube containing 0.5 ml 2% (v/v) glutaraldehyde. The grids were then dehydrated in a stepwise manner using 40, 60, 80, and 100% (v/v) ethanol. This was done by adding 1 ml of alcohol to the Eppendorf tube, gently mixing by pipetting the fluid up and down, and then removing all but enough to keep the grids from being exposed to the air. Each dehydration step was repeated once. The grids

were then transferred to a Tousimis Samdri 780 critical point dryer and critical point dried.

Etching

The ion etching was done in a modified Polaron model E5100 sputter coater. The apparatus has a 14 cm high by 15 cm wide vacuum chamber equipped with 14 cm diameter disk-shaped aluminum electrodes 4 cm apart. Critical point-dried grids were placed on the cathode, and the air was pumped out of the chamber. Ar gas was released into the chamber until a steady pressure of 100 mTorr was reached. A 5 mA current was then applied, ionizing the Ar which etched the grids on the cathode (figure 7). All of the grids to be etched were placed in the chamber at the beginning, with grids being removed at their accumulated time points. This lessened the possibility of heat damage to the grids by being exposed to the current for prolonged periods of time.

Shadowing and Observation

Following etching the grids were either rotary shadowed at a 70° angle, or unidirectional shadowed at a 30° angle, with Pt-Pd (80%:20%) and coated with carbon using a Balzers BAE 080 vacuum evaporator (Balzers, Hudson, NH). The grids were then observed on a JEOL 100cx electron microscope operated at 80 kV. Photographs were taken for further study and measurement.

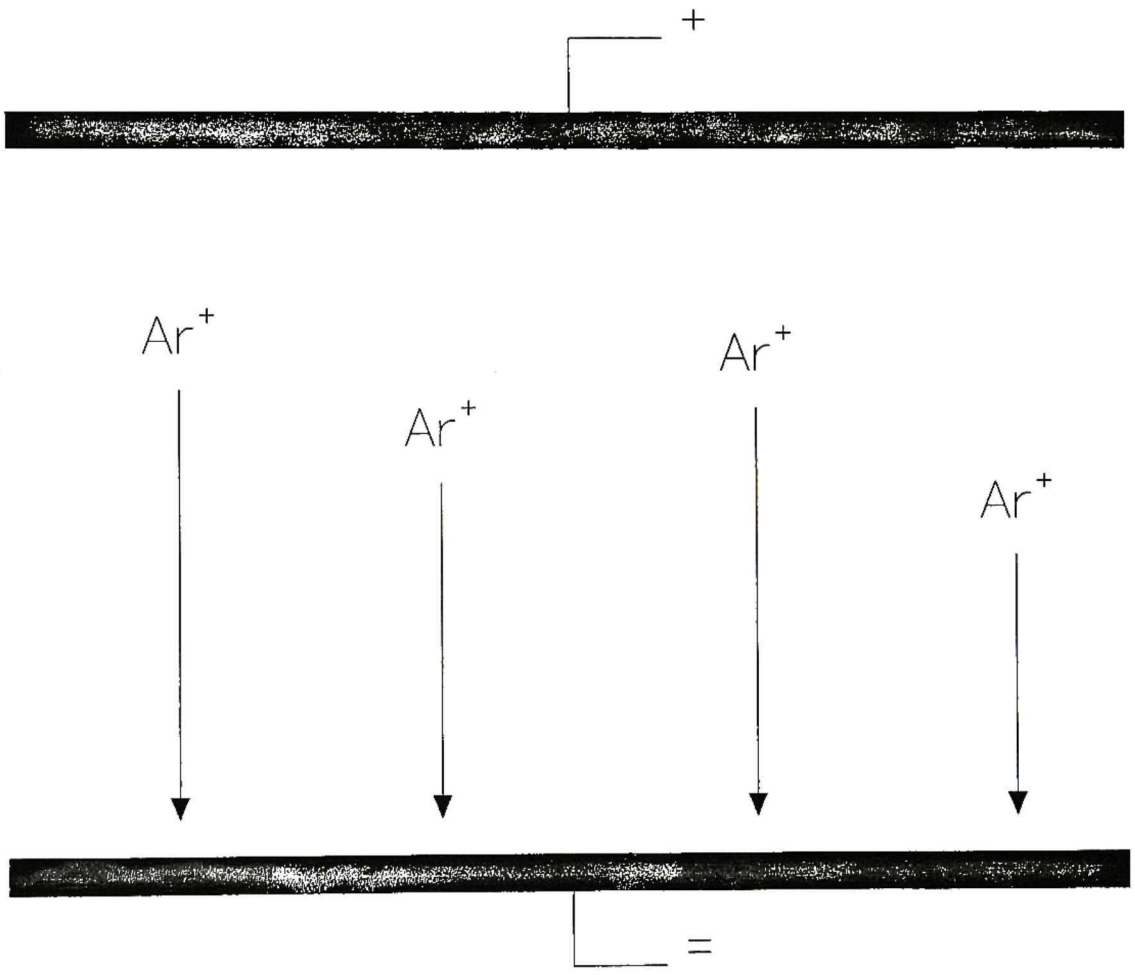
Molecular Cloning

Enzymes and Buffers

Enzymes and buffers used in the various cloning procedures were

Figure 7. A drawing representing the principle of ion etching. The ion etching apparatus consists of a vacuum chamber containing 2 electrodes. Specimens to be etched are placed on the cathode and the chamber is evacuated. Argon gas is leaked into the chamber to a pressure of 8 mTorr. An electrical current is applied ionizing the Ar which then bombards the cathode etching away the exposed layers of the specimen.

Ion Etching



purchased from: Boehringer Mannheim Biochemicals, Indianapolis, IN; Life Technologies, Inc. (BRL), Gaithersburg, MD; Stratagene, La Jolla, CA; New England Biolabs, Inc., Beverly, MA; or Promega, Madison, WI and used as directed by the various manufacturers.

Sequences

The DNA sequence of VZV was kindly provided by Dr. Andrew Davison, Institute of Virology, University of Glasgow, Scotland, prior to its publication (Davison and Scott, 1986). The sequences for pBluescript® SK (Stratagene), pUC9 (Messing, 1983), and pBR322 (Bolivar *et al.*, 1977) were available from the VecBase sequence database found in the GCG Sequence Analysis Software Package (Genetics Computer Group, University of Wisconsin Biotechnology Center, Madison, WI). The plasmid pSC11 (Chakrabarti *et al.*, 1985) sequence was kindly provided by B. Moss.

The IBI/Pustell DNA and Protein Sequence Analysis System (International Biotechnologies, Inc., New Haven, CT) was used to create restriction maps and lists of fragments produced from specific restriction enzyme digests of the clones we were working with. The sequence of a fragment being cloned could be inserted into the sequence for the plasmid it was being cloned into and this could be used for restriction analysis by the program.

The GCG sequence analysis programs (Devereux *et al.*, 1984) were used to translate DNA sequences to protein sequences. These protein sequences could then be used to generate hydrophobicity and immunogenicity charts, and compared with each other to look for regions of homology.

VZV DNA Libraries

Three VZV genomic libraries were available in Dr. Hay's lab for cloning. The Eco RI library was constructed by Dr. Tom Casey while he was a graduate student in Dr. Hay's lab (Casey, 1983). This library is inserted in the pBR322 plasmid (Bolivar *et al.*, 1977). The Kpn I clones were a gift from Dr. Andrew Davison and are in pAT153 (Davison and Scott, 1983). The Bam HI clones were constructed by Mr. Mark Wellman, a technician in Dr. Hay's lab, using p541.10 as a vector.

General Techniques

The plasmids used for cloning were grown up in LB broth with the appropriate antibiotic (ampicillin for the Bam HI and Eco RI clones and tetracycline for the Kpn I clones) and the plasmid DNA was isolated using alkaline lysis (Birnboim and Doly, 1979) and CsCl purification (Radloff *et al.*, 1967). DNA fragments were isolated by agarose-gel electrophoresis and either electroeluted or "genecleaned". Electroelution was done using an IBI electroelution apparatus (International Biotechnologies, Inc., New Haven, CT) following the manufacturer's instructions. "Genecleaning" was done using the Geneclean® Kit (BIO 101, Inc., La Jolla, CA) following the manufacturer's instructions. In this method the agarose is dissolved in sodium iodide and the DNA is bound to silica beads. The beads are washed several times and then the DNA is eluted into clean TE.

Transformation of DH5α® *E. coli* (BRL) was done with $CaCl_2$ precipitated DNA using the Hanahan (1983) technique. Other molecular cloning techniques unless otherwise noted, were done as described by Maniatis *et al.* (1982).

Vectors

pUC9

pUC9 is a 2.7 kb plasmid derived from pBR322 and M13mp9 (Messing, 1983). This plasmid contains the β-lactamase gene conferring ampicillin resistance upon its transformants. It also contains the sequence for the α-peptide of the *lacZ* gene (β-galactosidase). There is a multiple cloning site between the *lac* promoter and its gene providing blue/white color selection of clones (figure 8).

pBS and pBluescript® SK

pBS and pBluescript® SK (Stratagene), are both 3 kb phagemids derived from pUC19. They have a multiple cloning site, flanked by T3 and T7 promoters, inserted between the *lacZ* gene and its promoter, which allows for blue/white color selection of clones and *in-vitro* transcription of the cloned gene. The two phagemids differ only in the restriction sites found in the multiple cloning site (figure 9).

pSC11

pSC11 (Chakrabarti *et al.*, 1985) was kindly provided by Dr. B. Moss. This plasmid contains the thymidine kinase gene of vaccinia virus into which has been inserted the *lacZ* gene under the control of the vaccinia p11 promoter. Next to the p11 promoter is the vaccinia p7.5 promoter in the opposite orientation. Downstream from the p7.5 promoter is a unique Sma I site into which genes can be cloned under the control of this promoter (figures 10 and 11). When cells infected with vaccinia are transfected with this plasmid, homologous recombination can take place between the TK genes in the viral genome and the plasmid, thus inserting both the β-galactosidase gene and the cloned gene, under the control of

Figure 8. A map of the plasmid pUC9 showing the multiple cloning site inserted between the α portion of the Lac Z gene and the Lac I genes, and the β-lactamase gene which confers ampicillin resistance on cells containing the plasmid. DNA fragments inserted into the multiple cloning site block transcription of the Lac Z gene which blocks α-complementation.

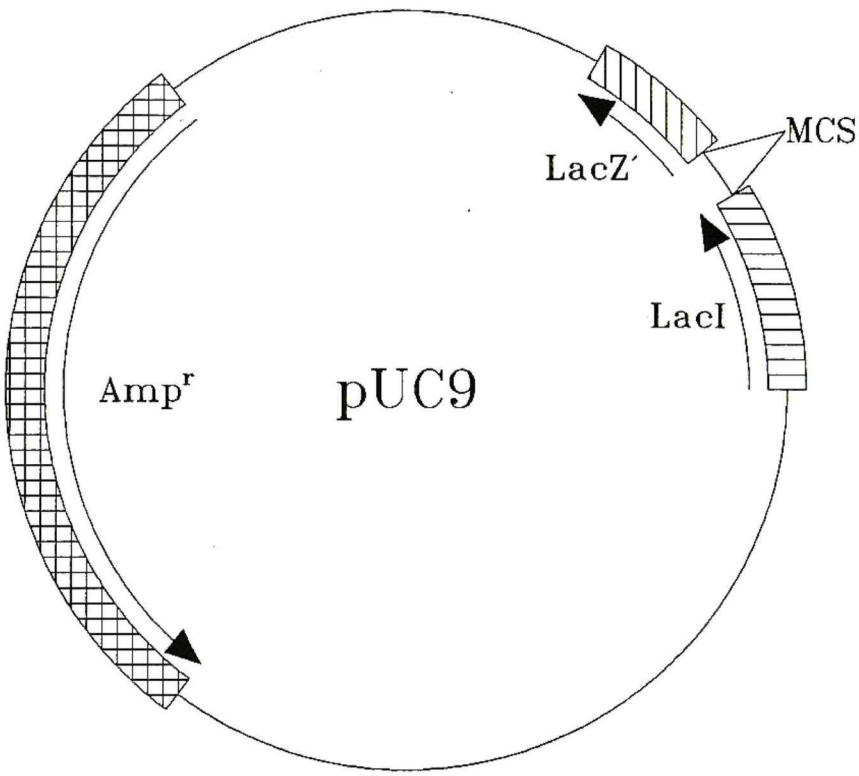

MCS
LacZ´
LacI
Amp^r
pUC9

Figure 9. A map of the phagemid pBluescript. This plasmid is similar to pUC in that it has ampicillin resistance and a multiple cloning site between the Lac I gene and the α portion of the Lac Z gene. The multiple cloning site is bounded by phage T7 and T3 promoters which allows inserts to be transcribed *in vitro*. It also contains an f1 origin of replication which allows single stranded DNA to be made for sequencing and site-directed mutagenesis.

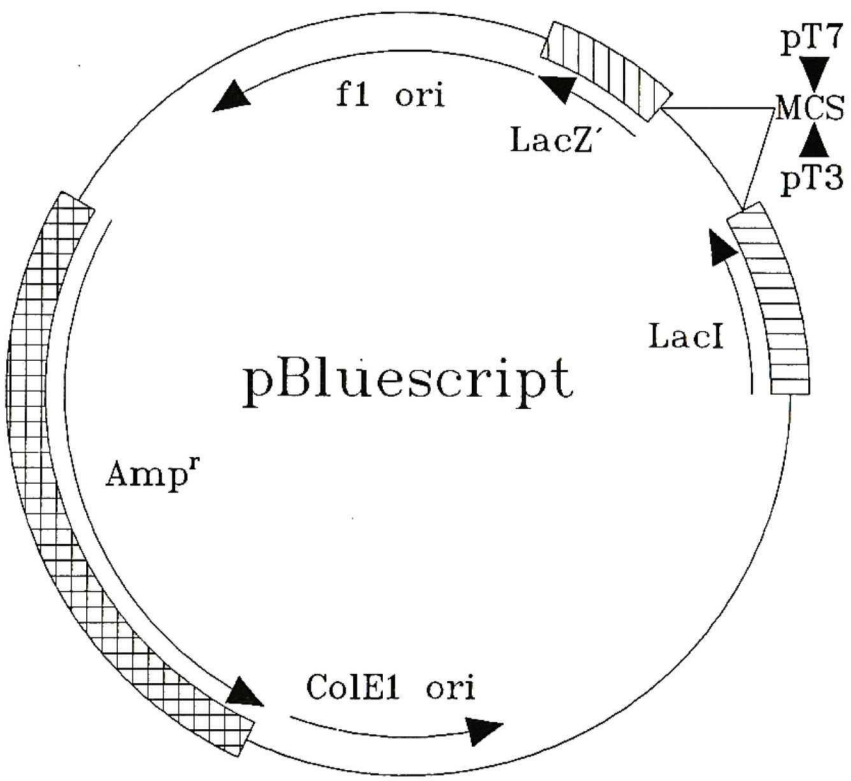

pT7
MCS
pT3
f1 ori
LacZ´
LacI
pBluescript
Amp^r
ColE1 ori

Figure 10. A map of the plasmid pSC11. This plasmid contains the vaccinia thymidine kinase gene into which has been inserted the β-lactamase gene under the control of the vaccinia p11 promoter, and the vaccinia p7.5 promoter directly upstream from a unique Sma I site.

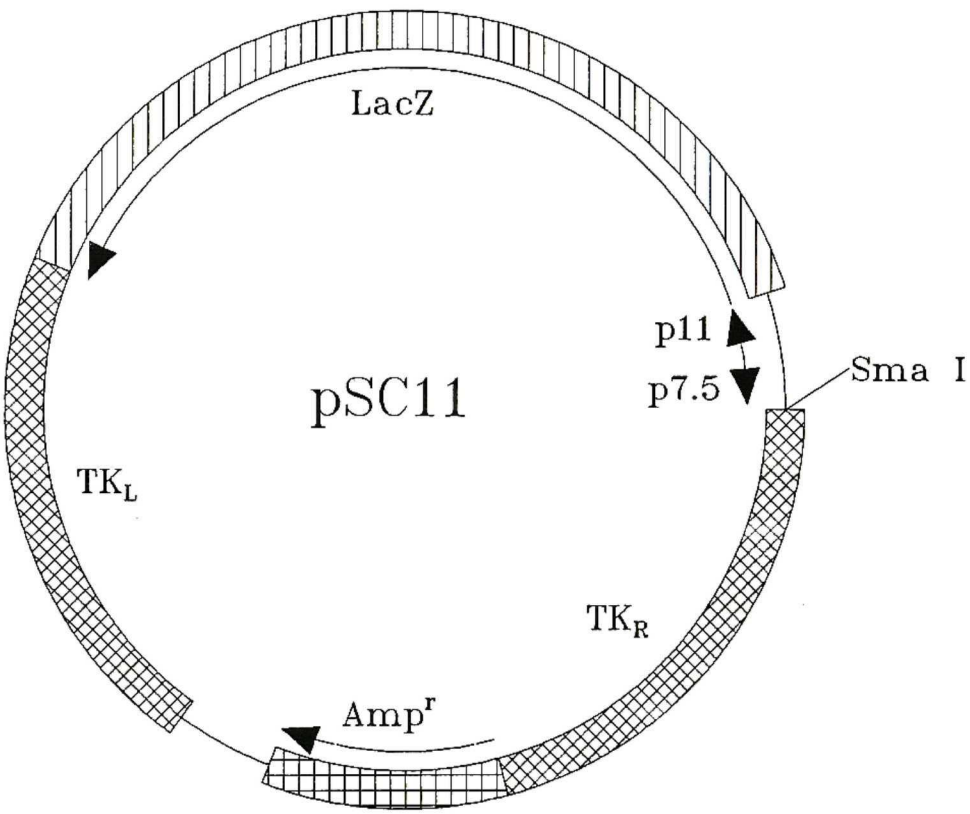
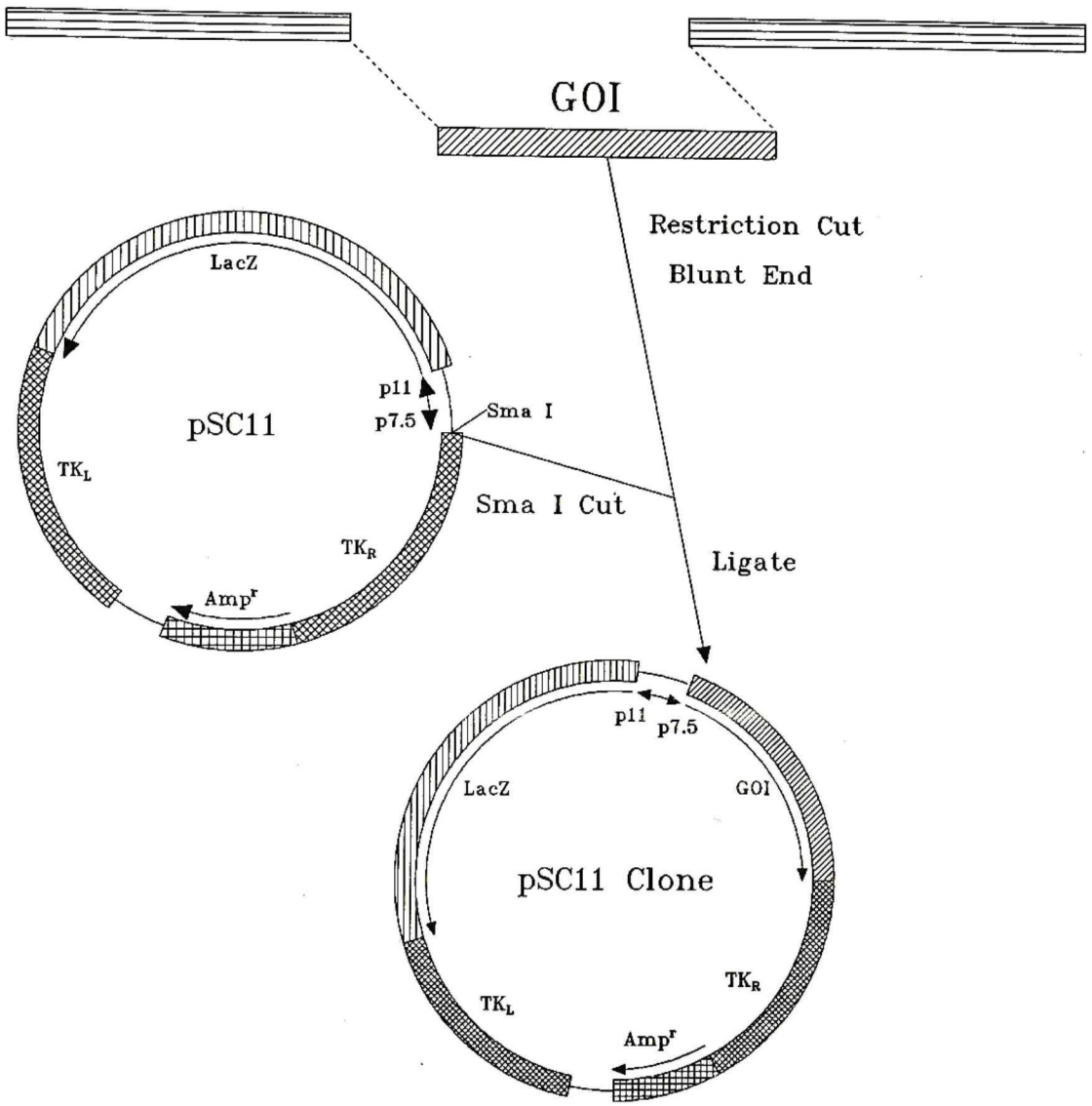

LacZ
p11
p7.5
Sma I
pSC11
TK$_L$
TK$_R$
Amp^r

Figure 11. A diagram of how genes are cloned into pSC11. The gene of interest (GOI) is cut out using the appropriate restriction enzymes, and is blunt ended. The fragment containing the gene is combined with pSC11 which has been cut with Sma I, and ligated into the plasmid. Clones are selected by probing colony blots with labelled DNA specific for the insert.


GOI
Restriction Cut
Blunt End
LacZ
p11
p7.5
pSC11
Sma I
TK$_L$
Sma I Cut
TK$_R$
Amp^r
Ligate
p11
p7.5
LacZ
GOI
pSC11 Clone
TK$_R$
TK$_L$
Amp^r

vaccinia promoters, into the genome of vaccinia (figure 12). These vaccinia recombinants produce blue plaques when grown in the presence of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside).

Constructs

VZV Gene 11

The 2,769 bp Bsp1285/Mlu I fragment from the KpnC clone contains VZV gene 11. This fragment was inserted into the Sma I site of pUC9 to give p9VZ11 (appendix, figure 54). The same fragment was also inserted into the Sma I site of pSC11 to give pVZ11SC (appendix, figure 55).

VZV Gene 33

The 2,058 bp Ban I/Dde I fragment of the KpnD clone contains VZV gene 33. This fragment was inserted into the Sma I site of pUC9 to give p9VZ33 (appendix, figure 56). The 2,058 bp Hind III/Eco RI fragment from p9VZ33 was then cloned into the Sma I site of pSC11 to give pVZ33SC (appendix, figure 57).

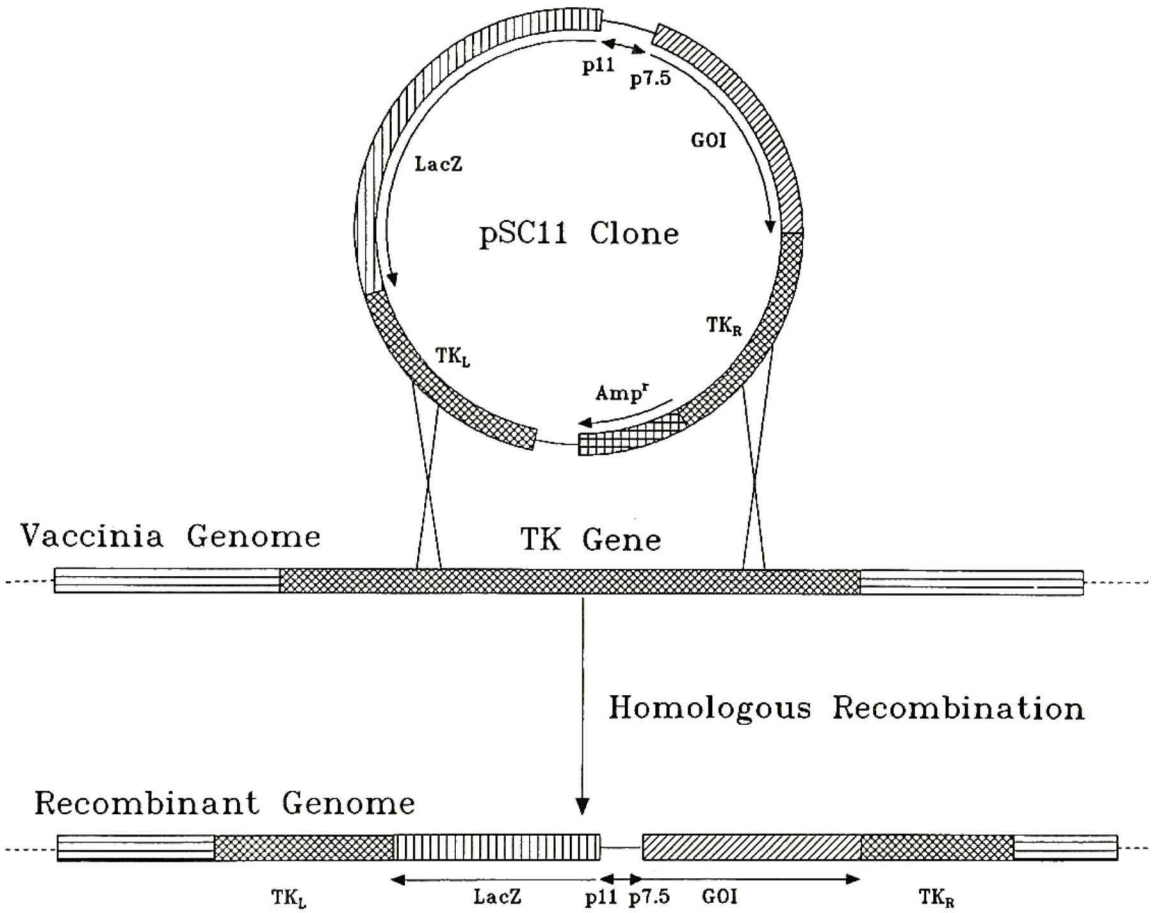
VZV Gene 34

The Cla I/Stu I 2,052 bp fragment of the BamH clone contains VZV gene 34. This fragment was inserted into both the Sma I site of pUC9, to give p9VZ34 (appendix, figure 58), and the Sma I site of pSC11 to give pVZ34SC (appendix, figure 59).

VZV Gene 40

The Bst XI 4,795 bp fragment of the EcoD clone contains VZV gene 40. This fragment has 4 ATG sites upstream from the actual translation

Figure 12. A diagram showing the insertion of a cloned gene into the vaccinia genome using pSC11. The plasmid is added to cells infected with the vaccinia virus. The cells take up the plasmid and homologous recombination can take place between the 2 halves of the vaccinia TK gene in the plasmid and the genomic TK gene. This inserts both the gene of interest and the β-lactamase gene into the vaccinia genome.


p11
p7.5
GOI
LacZ
pSC11 Clone
TK$_R$
TK$_L$
Amp^r
Vaccinia Genome
TK Gene
Homologous Recombination
Recombinant Genome
TK$_L$
LacZ
p11
p7.5
GOI
TK$_R$

start site. Therefore, in an unmodified form, it is not ideal for cloning into pSC11. It was nonetheless cloned into the pSC11 Sma I site to give pVZ40BSC (appendix, figure 60).

To get rid of the extra upstream ATGs, a two step procedure was used. First, the 275 bp Hpa II/Xba I fragment of the EcoD clone was inserted into pBS, which had been cut with Acc I and Xba I, to give pHX4 (appendix, figure 61). A Hpa II cut fragment may be inserted into an Acc I site with the loss of the restriction site. Next, the 4,906 bp Xba I fragment of EcoD was inserted into the Xba I site of pHX4 to give pHX4' (appendix, figure 62). The pHX4' Hind III/Eco RI 5,131 bp fragment was then cloned into pSC11 to give pVZ40HXSC (appendix, figure 63).

The multiple cloning site of pBS has an Sph I site upstream from its Acc I site. This site contains an ATG, therefore pVZ40HXSC had an extra ATG upstream from the actual translation start site. To remove this site, the 2,163 bp Pst I fragment of pHX4' was cloned into the Pst I site of pUC9 to give p9VZ40P (appendix, figure 64). The 3,751 bp Nae I/Eco RI fragment of pHX4' was then put into p9VZ40P, which had been cut with Nae I and Eco RI, to give p9VZ40 (appendix, figure 65). The 5,110 bp Hind III/Eco RI fragment of p9VZ40 was then cloned into the Sma I site of pSC11 to give pVZ40SC (appendix, figure 66).

VZV Gene 47

The 1,867 bp Hga I fragment of the BamG clone contains VZV gene 47. This fragment was inserted into both the Sma I site of pUC9 to give p9VZ47 (appendix, figure 67) and the Sma I site of pSC11 to give pVZ47SC (appendix, figure 68). The pVZ47SC 1,875 bp Bam HI/Mst II fragment was cloned into pBluescript® SK+ to give pVZ47BS (appendix, figure 69).

VZV Gene 66

Gene 66 was another gene that could not be cloned as a single fragment. The EcoA clone 974 bp Acc I fragment was first cloned into pBluescript® SK+ to give pVZ66ABS (appendix, figure 70). This was followed by the EcoA clone 1,839 bp Mlu I/Kpn I fragment inserted into pVZ66ABS cut with Mlu I and Kpn I to give pVZ66BS (appendix, figure 71). The 2,110 bp Hind III/Kpn I fragment from pVZ66BS was then cloned into the Sma I site of pSC11 to give pVZ66SC (appendix, figure 72).

Vaccinia Recombinants

Vaccinia recombinants were made using the techniques and vector (pSC11) described by Chakrabarti *et al.* (1985). The pSC11 clones of VZV genes 33, 34, 40, and 47 were successfully inserted into vaccinia virus in the following manner. Confluent CV-1 cells in 60 mm plates were infected with 0.5 ml/plate of vaccinia virus (strain WR) at an moi of 0.05 pfu/cell. Following a 2 hr. adsorption period at 37°C the cells were washed twice with MEM and overlaid with 0.5 ml/plate of $CaCl_2$-precipitated DNA (1 μg/ml pSC11 clone DNA, 19 μg/ml calf thymus DNA, 125 mM $CaCl_2$, in HEPES-buffered saline) and allowed to incubate at 37°C for 30 min. Next, 5 ml of MEM-10%SP was added to each plate and they were allowed to incubate for another 3.5 hr. at 37°C. The medium was then replaced and the cells were allowed to incubate for another 48 hr.

Following the incubation period, the infected cells were scraped into the media and disrupted by freeze/thawing and sonication. The disrupted cells were diluted in MEM and placed on 60 mm plates of confluent TK^- 143 cells. Dilutions of 1:2, 1:5, 1:10, 1:20, and 1:50 were

allowed to adsorb for 45 min. at 37°C. The plates were then overlaid with 4 ml/plate of 1% (w/v) low gelling temperature agar (LGA) in MEM with 25 μg/ml BUdR and allowed to incubate at 37°C for 48 hr.

The plates were next overlaid with 2 ml/plate of 1% (w/v) LGA in MEM with 0.01% (w/v) X-gal and allowed to incubate for another 24-48 hr. Blue plaques were then picked by aspirating an agar and cell plug with a micropipette and put on CV-1 cells to amplify the virus.

The infected CV1 cells were scraped into the media and disrupted by freeze/thawing and sonication to free the virus. The virus was diluted and again plaque purified by growing on TK^- cells in the presence of BUdR. This plaque purification/amplification cycle was repeated 3-4 times to insure the purity of the recombinant virus. Following purification, stocks were grown up and frozen down as described previously.

Four vaccinia recombinants: VZ33Vac, VZ34Vac, VZ40Vac (from pVZ40SC), and VZ47Vac were made in this manner. To insure that the VZV genes were intact in the vaccinia genome, DNA from each of the recombinants and wild type vaccinia was isolated in the following manner. CV1 cells infected with the vaccinia recombinants were scraped, pelleted, and resuspended in PBS. The cells were sonicated using the probe sonicator and layered onto a 5 ml 50% (w/v in gradient buffer) sucrose cushion and spun at 25,000 rpm in an SW-28.1 rotor for 1 hr. The pellet was resuspended in 1 ml of PBS, DNase was added to make a final concentration of 25 μg/ml, and then incubated for 1 hr at 37°C. Next, 20% (w/v) SDS was added to a final concentration of 0.5% (w/v) and proteinase K to a final concentration of 50 μg/ml and the samples were incubated for 1 hr. at 65°C. The DNA was phenol/chloroform extracted until no protein precipi-

tate was visible, and then ethanol precipitated (Maniatis *et al.*, 1982).

DNA from each recombinant, its respective pSC11 clone, and vaccinia (WR), was cut with Bam HI and Eco RI and electrophoresed on a 1% (w/v) agarose gel. The DNA was Southern blotted to Biodyne and the blots were probed with random primer-labelled DNA fragments from each of their respective pSC11 clones.

Antibody Production in Rabbits

The vaccinia recombinants were used to inoculate rabbits to obtain antibodies against the VZV gene products inserted into them. Each rabbit was inoculated intradermally with 0.5 ml of gradient purified virus at 2 X 10^8 pfu/ml. Each dose was split and injected into 3 sites on the rabbit's back. Injections were given at 2 wk. intervals. Prior to each injection, 10 ml of blood was taken from the ear vein of the rabbit. The serum from the test bleeds was stored at -20°C.

The two pBluescript® clones, pVZ47BS and pVZ66BS, were transcribed and translated *in vitro* by Dr. Michael Pensiero in Dr. Hay's lab. Bacteriophage T3 RNA polymerase has a high degree of specificity for its own promoter, therefore when genes are inserted directly downstream from the T3 promoter mRNA transcripts may be made (Melton *et al.*, 1984). The RNA transcripts made in this manner were put into rabbit reticulocyte lysate (Promega) for *in vitro* translation (Pelham and Jackson, 1976). A small portion of the reaction mix had ^{35}S-methionine (NEN) added so the translation products could be visualized following SDS-PAGE electrophoresis and autoradiography.

The *in vitro* translation mix was also given to rabbits in an attempt to make antibodies against genes 47 and 66. The translation mix

was combined with an equal volume of Freund's complete adjuvant and a 1 ml/rabbit initial inoculation was given to 2 rabbits for each gene. The rabbits were boosted with 0.5 ml/each 28 days later. Bleeds were collected at the time of injection and 2 wk. following each injection.

The rabbit sera were screened for antibodies against the VZV genes by western blot analysis against uninfected HFF cells, HFF cells infected with VZV, and purified VZV run on 10% (w/v) SDS-PAGE gels and transferred to nitrocellulose. The western blots were probed with a 1:50 dilution of rabbit serum in WB-2 with 1% (w/v) dried milk.

Protein Kinase Assays

In Vitro Virion Kinase Assay

VZV associated protein kinase (PK) activity was measured using a protocol based upon that of Strand and August (1971): Approximately 1 μg of purified virions was mixed with the reaction buffer (0.1% [v/v] NP40, 1.0 mM DTT, 50 mM Tris [pH 7.6], 30 mM $MgCl_2$, 15 μM ATP), which already had 2-3 μCi of γ^{32}P-dATP added, and the reaction mix was incubated for 30 min. at 37°C. Following incubation the reaction mix either had SDS-PAGE sample buffer added and was run on a 10% (w/v) SDS-PAGE gel, or was TCA-precipitated and counted on a scintillation counter. SDS-PAGE gels were dried on a Hoefer vacuum gel dryer (Hoefer Scientific Instruments, San Francisco, CA) and autoradiographed using Kodak XAR-5 film. The film was developed using a Kodak autoprocessor.

PK Extraction

A soluble extract of the PK activity of purified VZV was made using the technique of Stevely *et al.* (1985). Approximately 30 μg of

virions had NP40 and NaCl added to make final concentrations of 10% (v/v) and 0.6 M, respectively, in a 200 μl final volume. After 30 min. at room temperature the extraction was diluted 10 fold with TE and the particulate matter was pelleted by spinning at 10,000 X g for 2 hr. at 4°C. The supernatant was put into an Amicon Centricon 30 filtration unit and spun for 30 min. in an SS34 rotor at 6,000 rpm at 4°C. The retentate was diluted to 2 ml with TE and spun again for another 30 min. The retentate was diluted again to 2 ml with TE and spun for another hour. The retentate containing the PK activity was collected and stored at -80°C. Each of these filtration steps provided a 5-10 fold dilution of the NP40 and NaCl used to extract the PK activity from the virions.

Autophosphorylation

In order to identify the protein kinases associated with purified VZV, the autophosphorylation technique of Celenza and Carlson (1986) was used. The proteins were first electrophoresed on a 10% (w/v) SDS-PAGE gel. The gel was then soaked for 30 min. in WB-1 w/o methanol and the proteins electroblotted to Immobilon-P for 90 min. at 50 V in WB-1 w/o methanol, as in the western blot procedure already described.

The proteins on the membrane were denatured by soaking in 7 M guanidine, 50 mM Tris (pH 8.3), 50 mM dithiothreitol (DTT), and 2 mM EDTA for 1 hr. at room temperature. They were then renatured overnight at 4°C in 100 mM NaCl, 50 mM Tris (pH 7.5), 2 mM DTT, 1% (w/v) non-fat dry milk, and 0.1% (v/v) NP40.

The membrane was blocked with 5% (w/v) non-fat dry milk in 30 mM Tris (pH 7.5) for 30 min. at room temperature, and then put into 30 mM Tris (pH 7.5), 10 mM $MgCl_2$, 2 mM $MnCl_2$, with 50 μCi/ml γ^{32}P-dATP (NEN) for

30 min. at room temperature, for autophosphorylation to take place. The blot was dipped in 30 mM Tris (pH 7.5) (wash buffer) and then washed for 10 min. each: twice in wash buffer; once in wash buffer with 0.05% (v/v) NP40; once again with wash buffer; and once with 1 M KOH. The blot was then rinsed: once with 10% (v/v) glacial acetic acid; once with wash buffer; and once with water, and put to film using Kodak XAR-5 film and an intensifying screen.

III

RESULTS

The overall aims of this dissertation were to examine purified virions of VZV in a number of ways that would add to our knowledge of the structure of the virus. First, we planned to etch virions with Ar^+ in the expectation of revealing layers of virion organization and defining the VZV core. Next, we would clone into expression vector systems the genes for several VZV proteins which we deduced might be elements of the VZV particle. This was done for two reasons; first, we wanted to know about the genetic location and nature of the polypeptide products for these putative structural genes, and second, we hoped to glean useful data on the cellular location of the proteins and the immune response to them. Finally, having identified two potential protein kinase genes in VZV, we attempted to characterize them and correlate their activity with virion-associated protein kinase activity.

Purification of VZV Virions

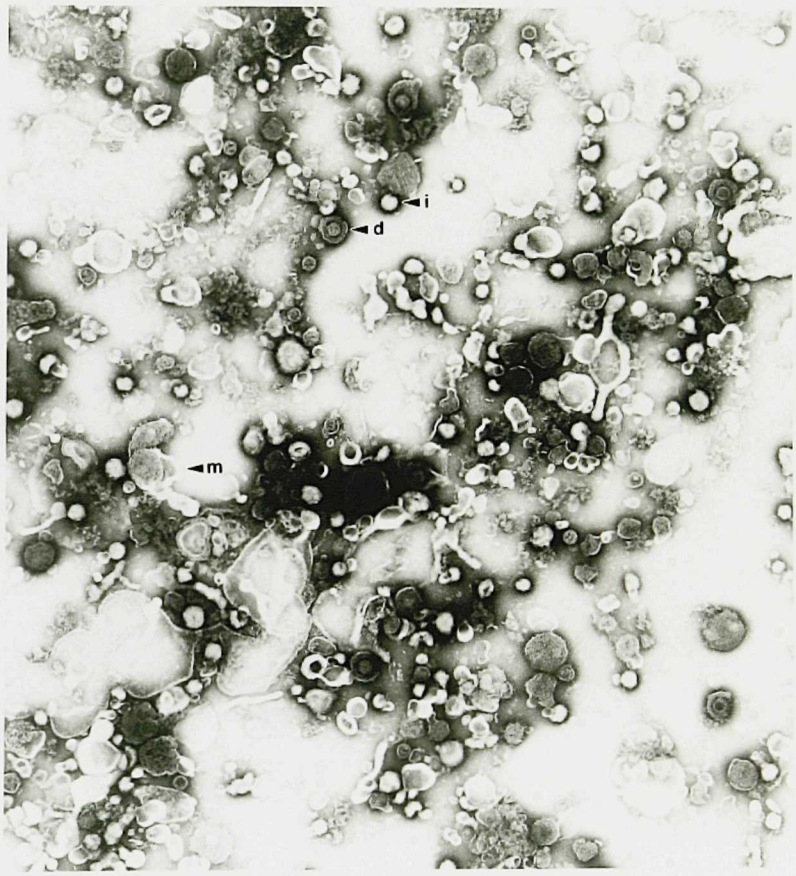
VZV, unlike HSV, is highly cell-associated in tissue culture (Taylor-Robinson, 1959). Cell-free VZV obtained from vesicle fluid is highly infectious (Weller and Stoddard, 1952), but very little infectious virus appears in the medium from infected cell cultures, and few virus particles can be recovered from this medium. In fact, work on the molecular biology and structure of VZV has been hampered by the inability

to obtain reasonable quantities of purified particles. In order to purify a sufficient quantity of virions for experimental purposes, they must be isolated from a cytoplasmic extract of infected cells (Shemer *et al.*, 1980). This increases the difficulty of purifying the virions because they must be separated from both non-structural viral proteins and cellular proteins and membranes.

There have been numerous descriptions of techniques for the purification of a number of herpesviruses, including HSV and EHV-1, for which excellent, rapid methods are available. Unfortunately, none of these methods appears to be applicable to the isolation of clean VZV. Historically, VZV virions have been isolated using two different centrifugation techniques, neither of which is completely effective on its own. The first involves a sucrose rate/zonal gradient of a cytoplasmic extract from infected cells (Shemer *et al.*, 1980). This step cleans away much of the contaminating material, but there is still a significant amount of extraneous membrane and protein present (figure 13). A second purification procedure (Obijeski *et al.*, 1974) takes cytoplasmic virus and applies it to a glycerol-tartrate gradient, in which the virus bands. We have combined the best features of these techniques into a method which gives good yields of VZV, free of host cell protein contamination.

Virions were collected from the sucrose gradient and applied to a glycerol/KT isopyknic gradient. Following this step two bands were seen (figure 6), the lower of which contained enveloped virions, while the upper band was a mixture of disrupted virions and nucleocapsids. When the material from the lower band of this glycerol/KT gradient was stained

Figure 13. An electronmicrograph of the material collected from a 20-50% (w/v in gradient buffer) sucrose rate/zonal gradient of the cytoplasmic extract from cells infected with VZV. The material was allowed to adsorb to formvar/carbon coated grids and stained with PTA. Many intact (i) as well as disrupted (d) virions can be seen along with cellular membranes and debris (m).



300 nm

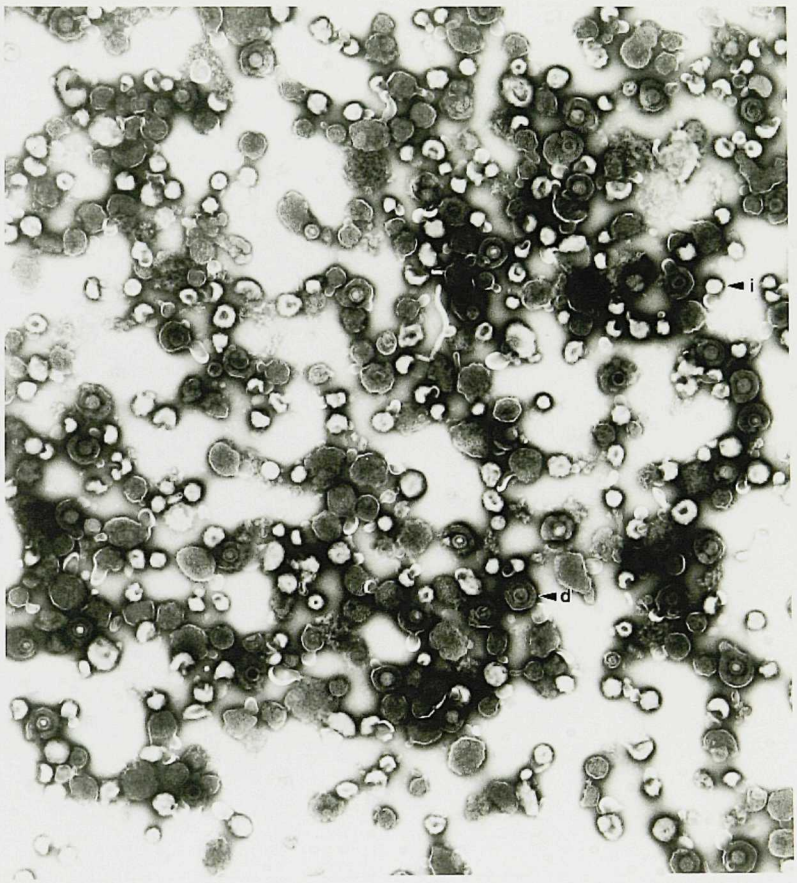
VZV Post Sucrose Rate/Zonal Centrifugation

with phosphotungstic acid and examined in the electron microscope, very little extraneous material was seen contaminating the virions (figure 14). On occasion this glycerol step was repeated and, while this resulted in somewhat cleaner virion preparations (as judged by electron microscopy), they appeared less intact than after just one glycerol/KT gradient (figure 15). The degree of purification after this second gradient, compared to the loss of integrity of the viral particles which resulted from it was not considered significant enough to warrant the use of this step in routine viral preparations.

In order to quantitate the cleanliness of the virions purified in this manner, cells labelled with ^{35}S-methionine were added to infected cells prior to the first step of the purification procedure. Following each step of the procedure samples were taken and their protein concentrations and radioactivity were measured. From these data the cpm/μg of protein were calculated, and the percentage radioactivity remaining following each step was assessed relative to the cellular homogenate. The data were plotted in figure 16. The cytoplasmic extract removed more than half of the labelled host cell protein, with a 41.26% reduction in cpm/μg of protein. The sucrose gradient almost doubled this with a 74.55% reduction, and the first glycerol/KT gradient lowered the relative percentage of cellular contaminating counts remaining to 1.67%. The final glycerol/KT gradient dropped this further to 0.74% of the cpm/μg of protein of the original cellular homogenate, a 135-fold purification overall.

A more direct means of looking at the purity of the virion preparations, on a day to day basis, was to look for contaminating

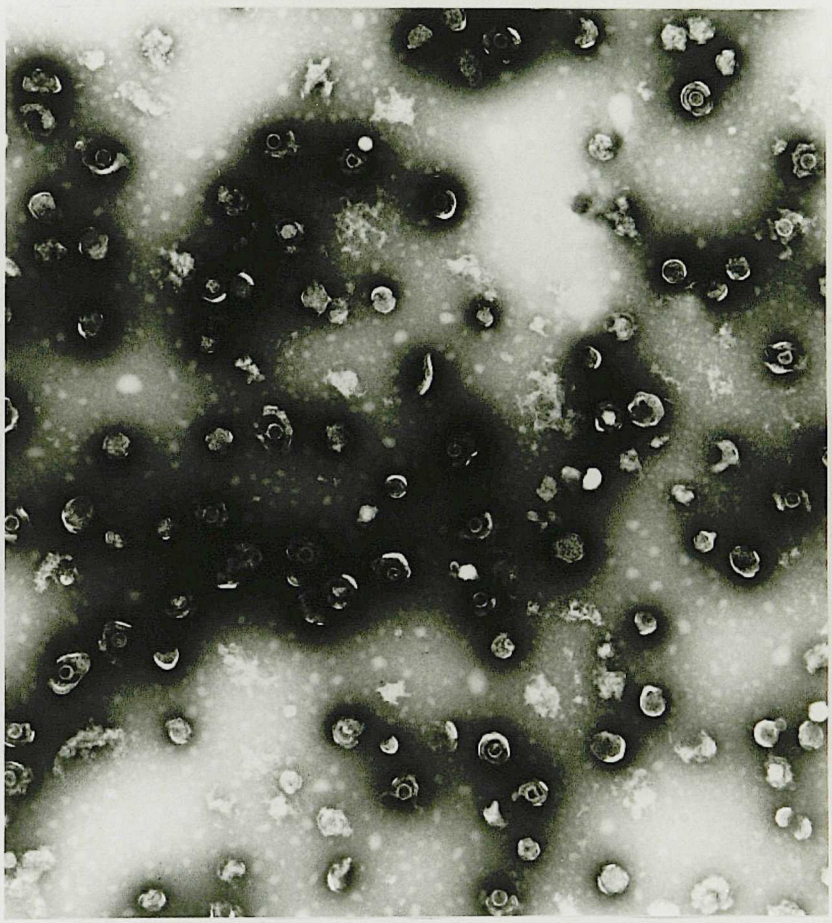
Figure 14. An electronmicrograph of the virions harvested from a glycerol/KT gradient. The virions were adsorbed to formvar/carbon coated grids, stained with PTA, and viewed as in the previous electronmicrograph. The virions are quite clean, with many of them still possessing intact membranes which excludes the negative stain, giving them a snowball-like appearance (i). Those virions which allow the stain to penetrate the membrane have the typical "fried egg" appearance associated with herpes virions (d).



300 nm

VZV Post Glycerol/KT Density Centrifugation

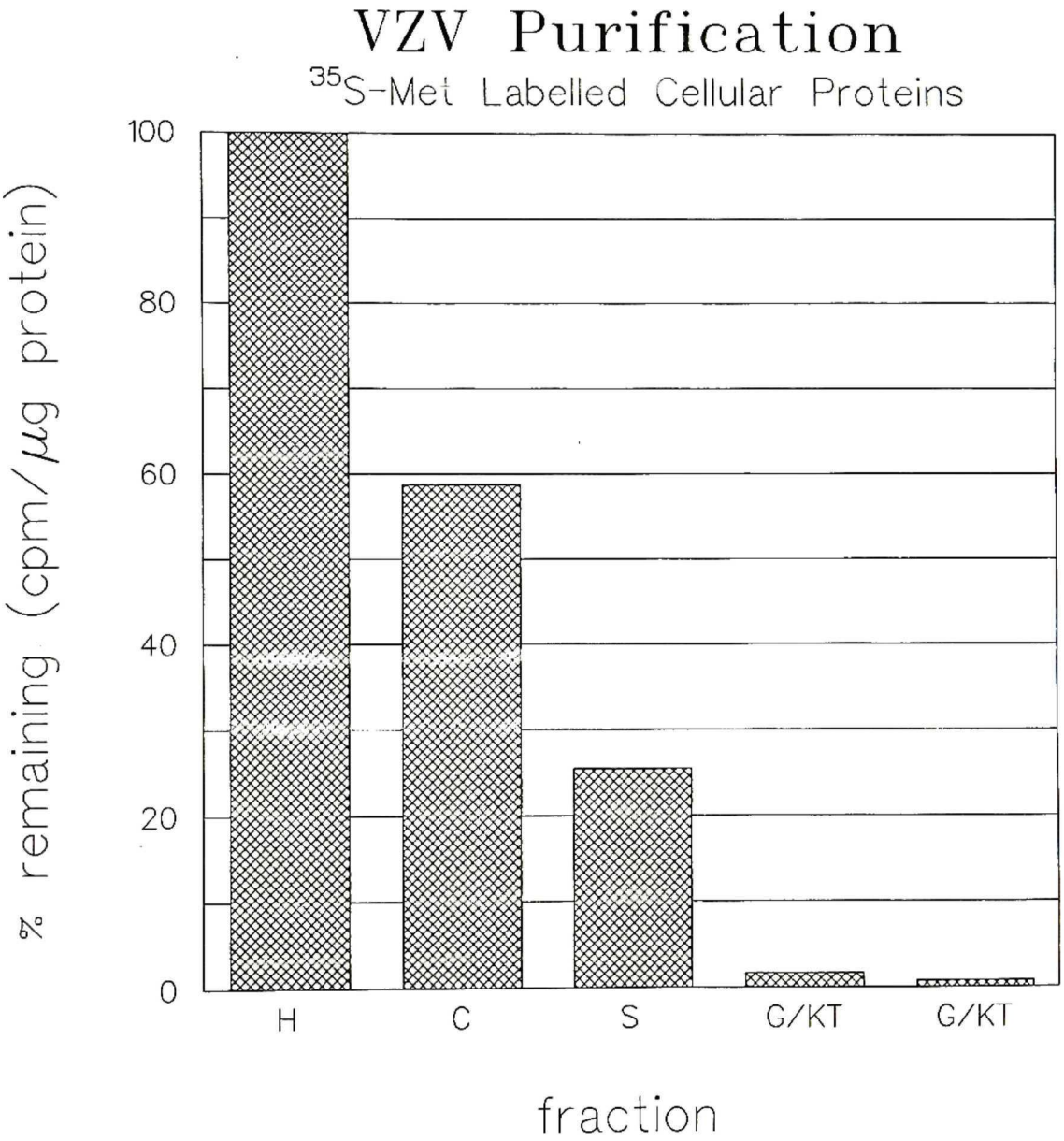
Figure 15. An electronmicrograph of virions following a second glycerol-/KT gradient to further purify them. The virions were stained and viewed as previously described. Most of the virions now have disrupted membranes, and in general, do not appear to be in very good condition.



300 nm

VZV Post Glycerol/KT Density Centrifugation

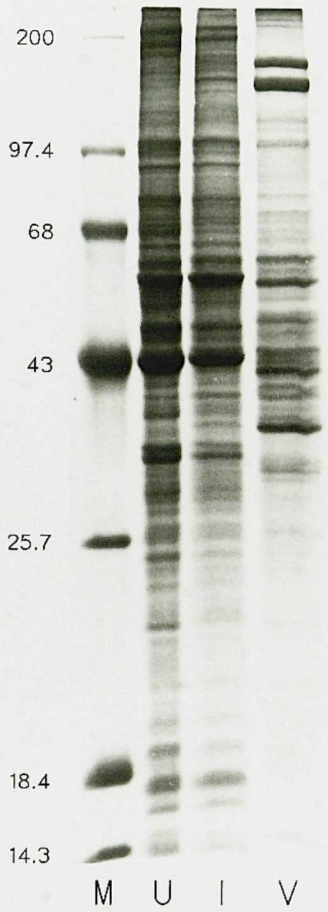
Figure 16. A histogram of the percentage of counts remaining in each fraction from the purification of VZV virions from cellular proteins labelled with ^{35}S-methionine, relative to the cellular homogenate. Labelled cells were added to VZV infected cells and homogenized (H). The nuclei were pelleted, and the cytoplasmic fraction (C) was layered onto a sucrose rate/zonal gradient. The virions harvested from the sucrose gradient (S) were layered onto a glycerol/ KT gradient. The virions harvested from this gradient (G/KT) were once again put onto a glycerol/KT gradient for final purification. These virions are represented by the final column (G/KT).


VZV Purification
35S-Met Labelled Cellular Proteins
% remaining (cpm/μg protein)
100
80
60
40
20
0
H
C
S
G/KT
G/KT
fraction

cellular proteins using CBB-G250 stained SDS-PAGE gels; CBB-G250 is a particularly sensitive protein stain when used as described in the materials and methods section, whose effectiveness is similar to that of a silver stain. The proteins of the virions were compared with those of both uninfected and infected cells (figure 17). There are at least 38 viral structural proteins present in this gel ranging in size from 218 to 13 kDa. It can be seen in this figure that the major cellular proteins are excluded from the virions, while at the same time viral proteins, seen in the infected cells, are concentrated in the virions. There are, of course, instances where viral proteins appear to migrate at the same point in the gel as cellular proteins, which leads to some ambiguity, but the clear-cut cases of cellular protein exclusion along with the data from the isotope exclusion experiment leads us to believe that any cellular proteins present in the virions are there only in trace amounts.

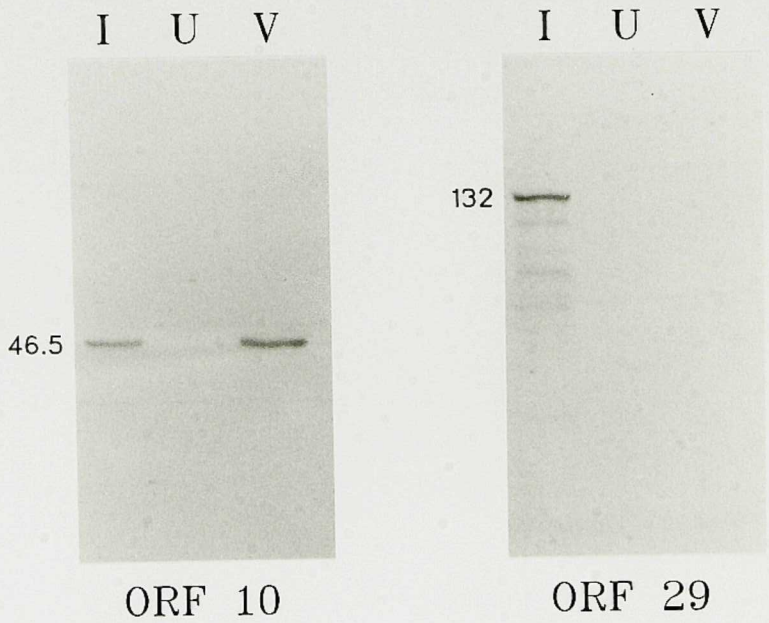
An important factor in the purification of the virions was to have clean enough virions to be able to distinguish between structural and non-structural proteins on the western blots we intended to do. Figure 18 is an example of western blots further demonstrating the efficacy of our technique. Rabbit antibody against a keyhole-limpet hemocyanin (KLH) conjugated synthetic peptide of the amino terminus of ORF10, the homologue of HSV αTIF which is a structural protein, gives bands in both infected cells and virions, while rabbit antibody against a KLH conjugated synthetic peptide of the carboxy-terminus of ORF 29, the homologue of HSV ICP8 which is a known non-structural protein, only lights up the infected cell lane. The antibody for these western blots was kindly provided by Dr. Paul Kinchington.

Figure 17. A photograph of a 10% (w/v) SDS-PAGE gel of proteins from HFFs (U), HFFs infected with VZV (I), and purified VZV virions (V). BRL high molecular weight protein markers are in lane M. Samples were run through the stacking gel at 15 mA and through the separating gel at 30 mA until the phenol red dye front reached the bottom of the gel. The gel was stained with Coomassie Brilliant Blue G250.



CBB-G250 Stained SDS-PAGE Gel

Figure 18. Photographs of western blots demonstrating the purity of the VZV virions. HFFs infected with VZV (I), HFFs (U), and purified virions (V) were run on 10% SDS-PAGE gels and blotted to nitrocellulose. The blots were probed with a 1:100 dilution of rabbit antisera against synthetic peptides of the carboxy terminus of the VZV ORF10 gene and the VZV ORF29. The ORF10 is a homologue of the HSV αTIF gene which codes for a known structural protein while ORF29 is a homologue of HSV ICP8 which codes for a known non-structural protein.



VZV Structural and Non-Structural Proteins

Structural Characterization of VZV Using Ion Etching

The herpesvirus virion has four main structural components; envelope, capsid, DNA-protein complex (core), and tegument. The envelope is a trilaminar membrane, derived from the host cell's nuclear membrane, and has spikes on its surface corresponding to viral glycoproteins. The capsid represents the most characteristic structural feature of the *Herpesviridae* family, with 150 hexavalent and 12 pentavalent capsomeres in a typical icosadeltahedral organization. In the intact virus, the capsid surrounds the core, and recent data from density studies of electron micrographs of frozen hydrated HSV nucleocapsids have suggested that there is a second icosadeltahedral shell between the capsid and the DNA mass (Schrag *et al.*, 1989). The tegument remains a poorly-defined structure lying between the capsid and the envelope, and perhaps plays a role in herpesviruses analogous to that of the matrix protein in some RNA viruses. It has no obvious structural characteristics and its size seems to vary markedly among the herpesviruses. The structure of the core is the subject of considerable controversy, in that several different models currently exist in the literature. Researchers have only been able to visualize the core using electronmicrographs of thin sections of either infected cells or purified virions. No one has been able to visualize the intact core. This has lead to several different interpretations of the data and the proposal of several models for the structure of the core (Nazerian *et al.*, 1971; Okada *et al.*, 1972). Much of this has probably been due to observation of the core at different stages of maturation (Perdue *et al.*, 1976). The most widely-accepted is the "plug and toroid" construction of Furlong *et al.* (1972), in which the viral DNA is present

in a doughnut shaped nucleoprotein approximately 50 nm high and 70 nm in diameter, inside which is a cylindrical plug, presumably made of protein.

Newcomb *et al.* (1984) have described the use of "ion etching" to visualize and define the core structure of adenovirus, which they found to be composed of twelve spheres, called adenosomes, each with contact to the vertices of the capsid icosahedron. In order to approach a direct definition of the core structure of VZV, we decided to employ this "ion etching" method to VZV virions.

Whole VZV virions were purified as outlined in materials and methods, adsorbed to formvar/carbon coated grids, fixed, critical point dried, and etched with Ar^+ for several different periods of time as described in the materials and methods section. Several grids were etched for different periods of time to establish appropriate time scales for the etching process. Following etching, the particles were shadowed and viewed with an electron microscope. Photographs of the etched particles were taken for each time point. Both the appearance and size of etched particles was noted at each time point of etching. Two photographs were randomly picked from each time period and 100 particles from each photo were measured and the geometric mean of their diameters was determined. This data was analyzed statistically using CoStat statistical software (Cohort Software, Berkeley, CA). In the first set of photos all particles present were measured, including clusters of particles and what appeared to be remnants of tegument without capsids. This caused the results to be skewed a bit toward larger sizes and a decrease in the apparent rate of etching. The second set of data which excluded clusters and tegument remnants had a more even distribution of particle sizes.

Following removal of those measurements identified as being clusters (28 out of 600 measurements) the statistical analysis of the first set of data was repeated and compared with the second set. The analysis of variance F test gave a value of 192.96 for the first set of data and 180.93 for the second, with both having a value of $P < .001$, indicating a highly significant difference between the mean diameters of the different time points. The Student-Newman-Keuls test was then used to calculate a least significant difference between the means of the different time points of 6.25 for the first and 6.3 for the second. These values show that there was no significant difference between the 10 and 20 sec. time points, but there was a significant difference between all the others for both sets of data.

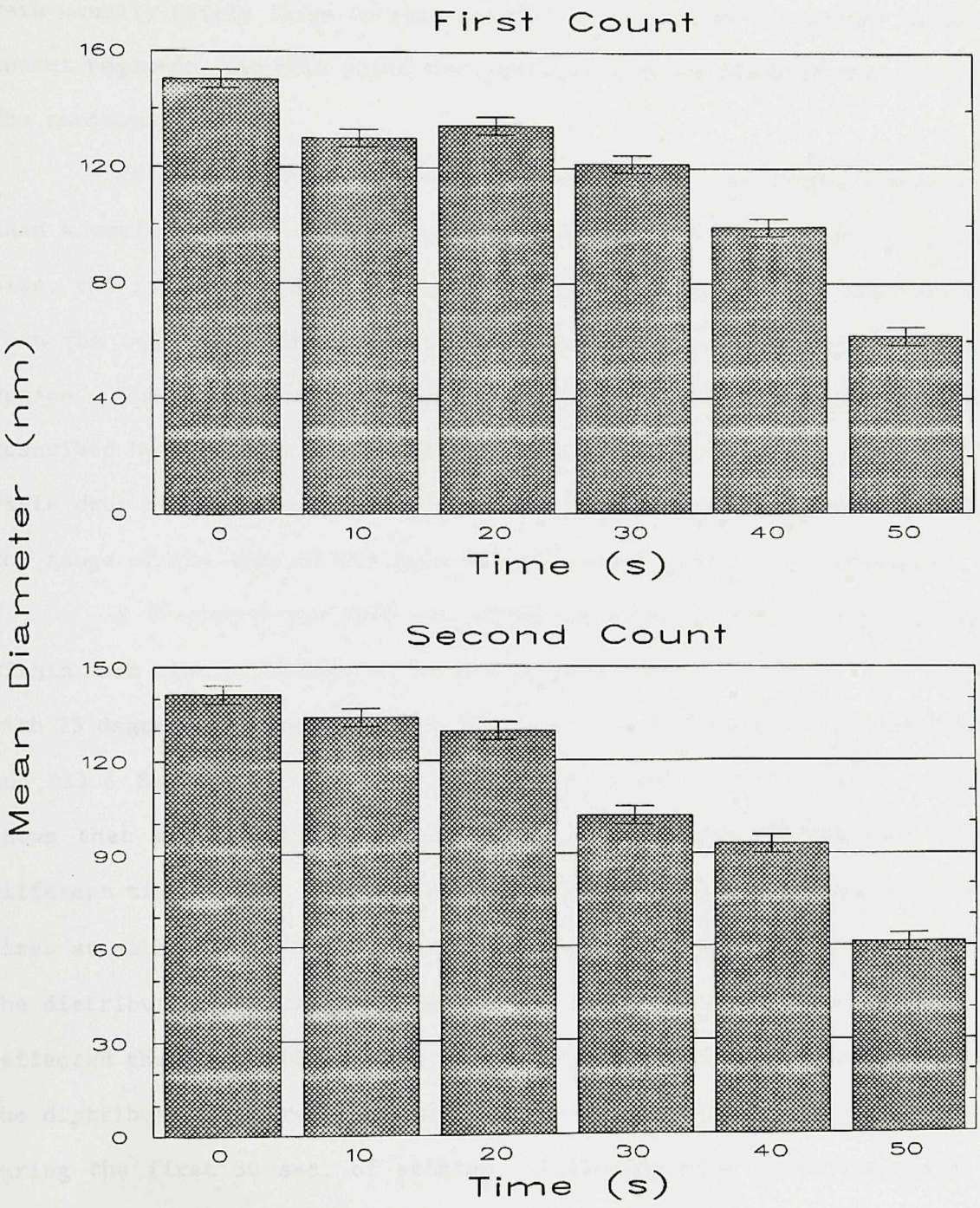
Figure 19 shows the mean diameter, plus or minus 2 standard errors of the mean of the particles at each time point. The sizes of the particles before etching ranged in size from 103-208 nm with the average being 150 nm for the first set and 141 nm for the second. After 10 sec. of etching there was a significant drop in the size of the particles which was probably due to the removal of the viral membrane and its associated glycoproteins.

At 20 sec. of etching the range of particle sizes was much the same as at 10 sec., with a non-significant drop in mean diameter. This lack of significant change in size suggested that the tegument was relatively resistant to the etching action of the Ar^+, implying the possibility of a tight, highly organized structure, possibly being held together due to the fixation step prior to etching.

After 30 sec. of etching, there was once again a significant drop

Figure 19. Histograms of the mean diameter, plus and minus 2 standard errors of the means of the population, of the particles at each stage of the ion-etching of the VZV virions. Two sets of measurements were performed as described in the text.

VZV ION ETCH


First Count
160
120
80
40
0
0
10
20
30
40
50
Time (s)
Second Count
150
120
90
60
30
0
0
10
20
30
40
50
Time (s)
Mean Diameter (nm)

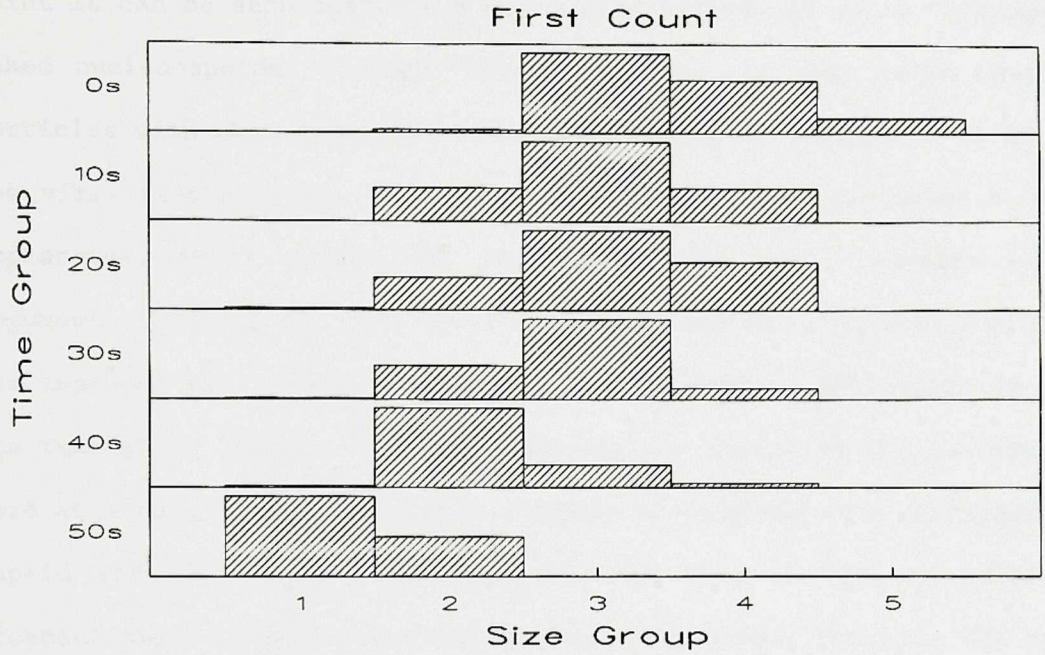
in the size of the particles. At his time point the two sets of data differed in the rate of decrease in particle size. This was probably due to the measurement of the disrupted teguments in the first, since these were usually fairly large in size and did not etch away as quickly as the intact tegument. At this point the particles were approaching the size of the nucleocapsid.

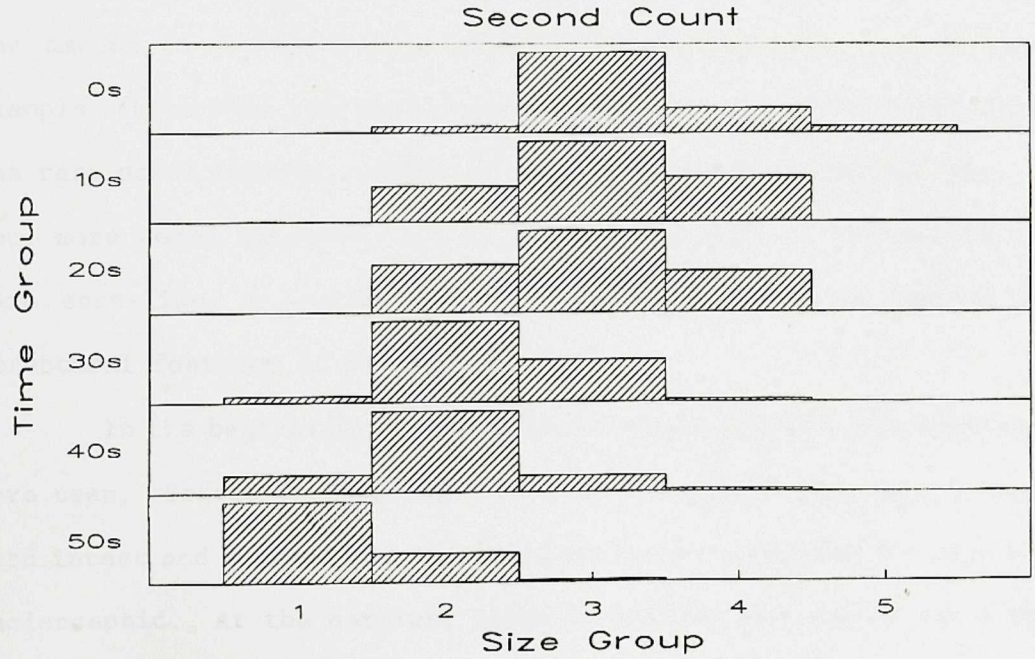
At 40 sec. both sets of data had an average size slightly smaller than a nucleocapsid. Even though there was again a significant drop in size, the rate of decrease in diameter had once again slowed indicating that the ions were having to etch through a more compact tightly bound region. This may have been due to the presence of the internal capsid described by Schrag *et al.* (1989). Finally, at 50 sec. there had been a rapid drop in particle size to the point where the majority fell within the range of the size of the core of the virus (about 70 nm diameter).

A frequency analysis was performed which divided the particles within each time point into 40 nm size ranges. Using this data, a χ^2 test with 25 degrees of freedom gave a value of 561.4 for the first set of data and 523.6 for the second, with both having a value of $P < .001$. This shows that the particle sizes were not randomly distributed over the different time points. A graph of the frequency analysis of the particle sizes at each time point better revealed what was happening (figure 20). The distribution of sizes at time 0 was a fairly normal distribution and reflected the variability of the amount of tegument found in each virion. The distribution progressively shifted toward the size of a nucleocapsid during the first 30 sec. of etching. Following this there was a progressive shift toward the size of the core over the next 20 sec.

Figure 20. Histograms of both sets of counts showing the size distribution of the particles seen at each time period during the ion-etching of the VZV virions. Each column represents a 40 nm range in particle sizes starting with 30-70 nm for the first column. No particles were seen below 30 nm or above 230 nm.

VZV ION ETCH

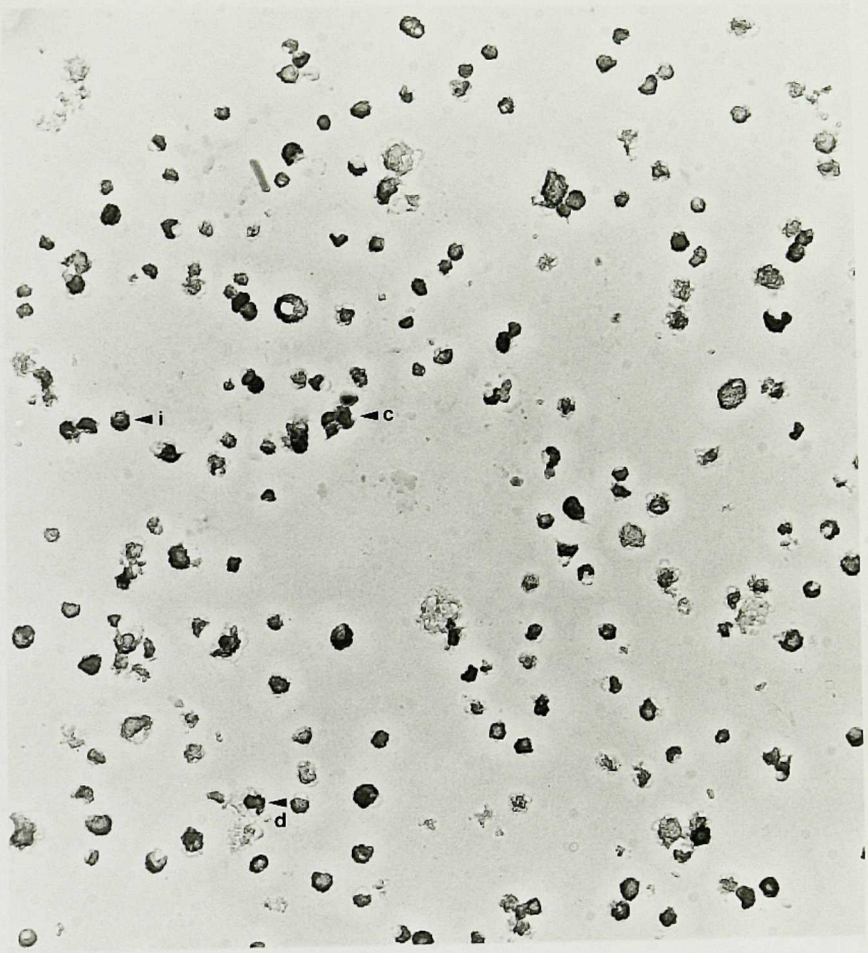

First Count
Time Group
0s
10s
20s
30s
40s
50s
1
2
3
4
5
Size Group


Second Count
Time Group
0s
10s
20s
30s
40s
50s
1
2
3
4
5
Size Group

Figures 21-33 are photographs of the viral particles showing typical examples at different time points of etching. At the 0 sec. time point it can be seen that there was a wide variety of sizes varying from naked nucleocapsids, through "normal" virions, to very large enveloped particles with what appeared to be an abundance of tegument. At 10 sec. the viral membrane had been etched away, giving the particles a smooth appearance, which presumably represented the outer surface of the tegument. Through the next two time points, the Ar^+ ions were bombarding the tegument and gradually loosening the structure sufficiently to allow its removal by 30 sec. Finally, the various layers of the nucleocapsid were attacked, leaving initially a number of doughnut or disk-shaped sub-capsid structures which were probably the core of VZV and eventually crescent-shaped structures which may represent broken toroids. The variability in time for the appearance of the cores appeared to be relative to the amount of tegument associated with each particle. At 30 sec. for example, there were rare core-like particles present which would represent the rare naked capsids present at the beginning being etched away. At 40 sec. more cores appeared, and at 50 sec. the bulk of the particles seen were core-like, a progression compatible with stepwise removal of the structural features of the VZV virion.

In the beginning, characteristic virion and sub- virion structures were seen. Before etching began (figure 21), there were many examples of both intact and damaged VZV enveloped particles revealing the tegument and nucleocapsid. At the earliest times of etching (10 and 20 sec.) many of the particles were smooth, tegument-associated capsids. At 30 and 40 sec., capsid sized structures were seen, but due to the presence of

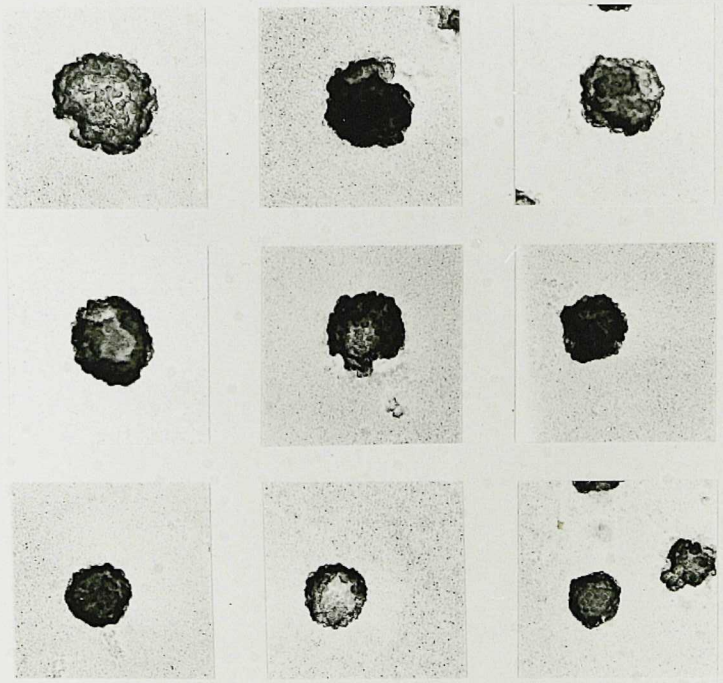
Figure 21. An electronmicrograph of the viral particles prior to being etched. A wide range of sizes and states of the particles can be seen, as in the negatively stained virions in fig. 14. Most of the virions are intact (i), but there are examples of disrupted virions (d) showing the tegument spread out and, in the case of the particle pointed out, the nucleocapsid exposed. Also seen are clusters of virions (c) which were not measured for the second set of measurements.



300 nm

VZV Virions Etched 0 Seconds

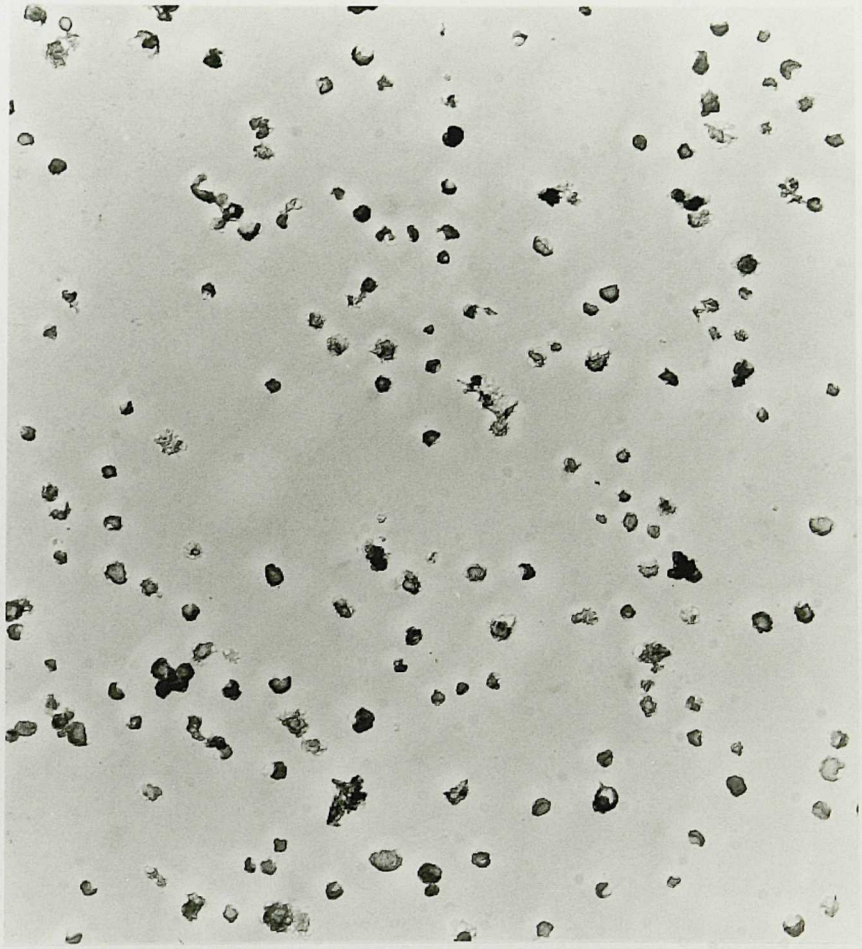
Figure 22. Selected viral particles, prior to etching, shown at a higher magnification. Note that the particles range from very large intact virions (upper left) to naked nucleocapsids (lower right).



100 nm

VZV Virions Etched 0 Seconds

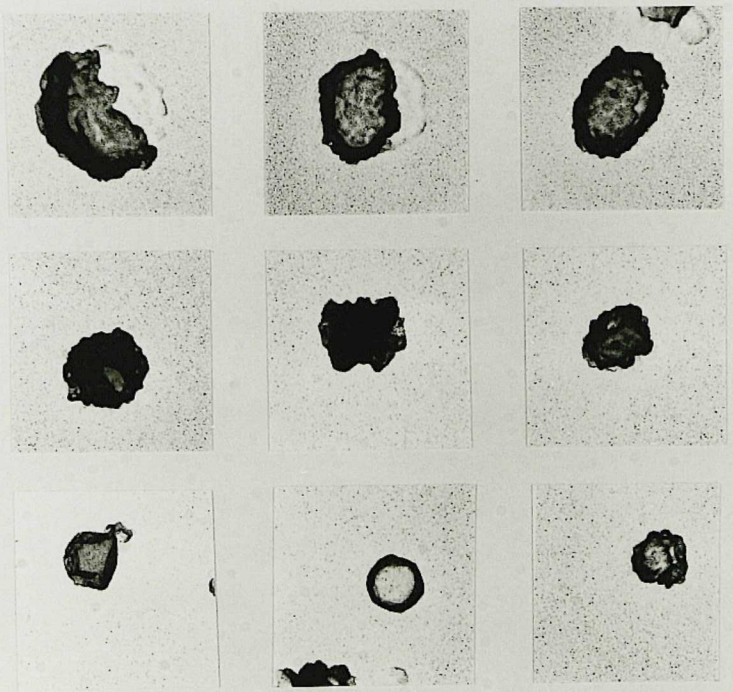
Figure 23. An electronmicrograph of virions following etching for 10 sec. Notice that the particles had a smoother appearance than the unetched virions.



300 nm

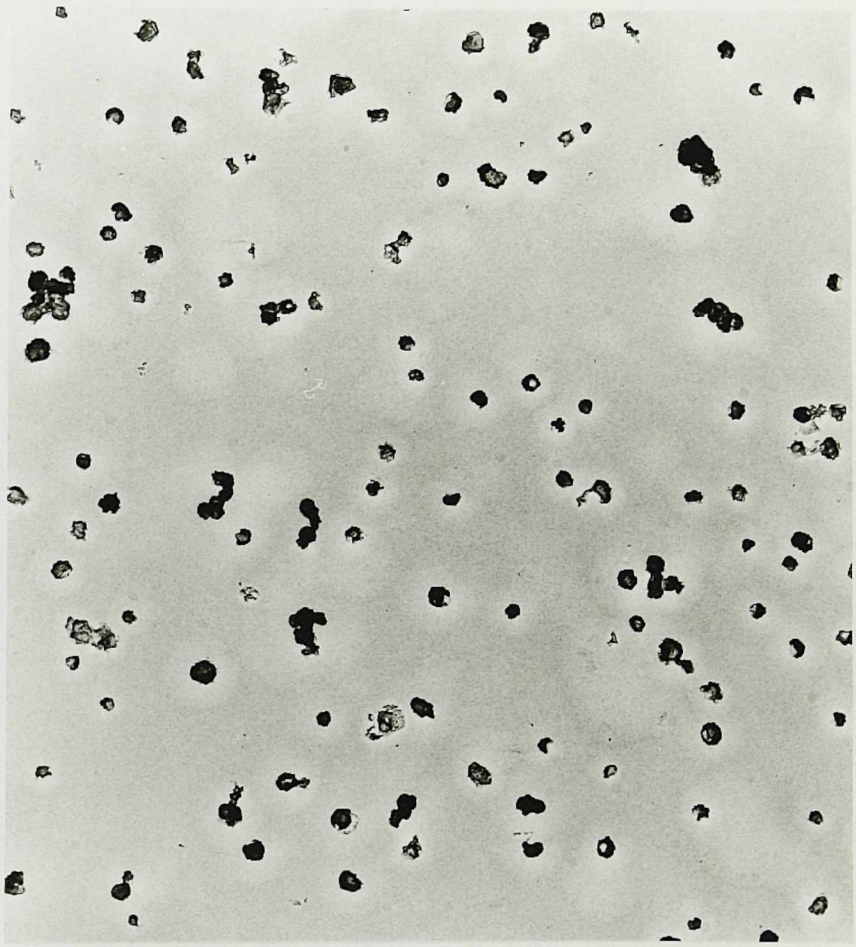
VZV Virions Etched 10 Seconds

Figure 24. Selected particles, at a higher magnification, following 10 sec. of etching. The smoother appearance was more evident than in fig. 23. Again particles ranged in size from large virions to nucleocapsids.



VZV Virions Etched 10 Seconds

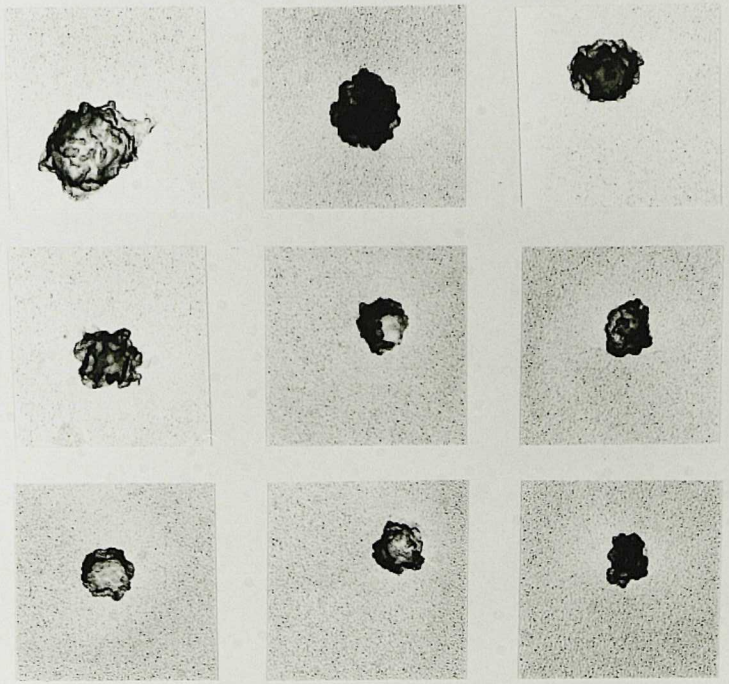
Figure 25. An electronmicrograph of virions following 20 sec. of etching. There was little change when compared with fig. 23.



300 nm

VZV Virions Etched 20 Seconds

Figure 26. Selected particles following 20 sec. of etching, at a higher magnification. The overall size of the particles decreased, and it was apparent that bits had been etched out of their surfaces.



100 nm

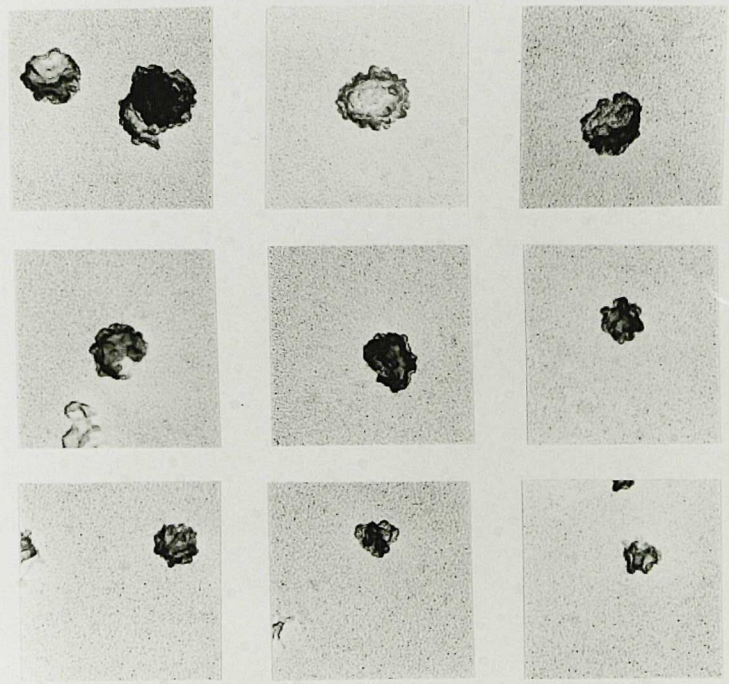
VZV Virions Etched 20 Seconds

Figure 27. An electronmicrograph of virions following 30 sec. of etching.

300 nm

VZV Virions Etched 30 Seconds

Figure 28. Selected particles following 30 sec. of etching, at a higher magnification. The last particle in the 2nd row and all of the particles in the bottom row were smaller than the size of the nucleocapsid and showed the etching away of chunks of the capsid.



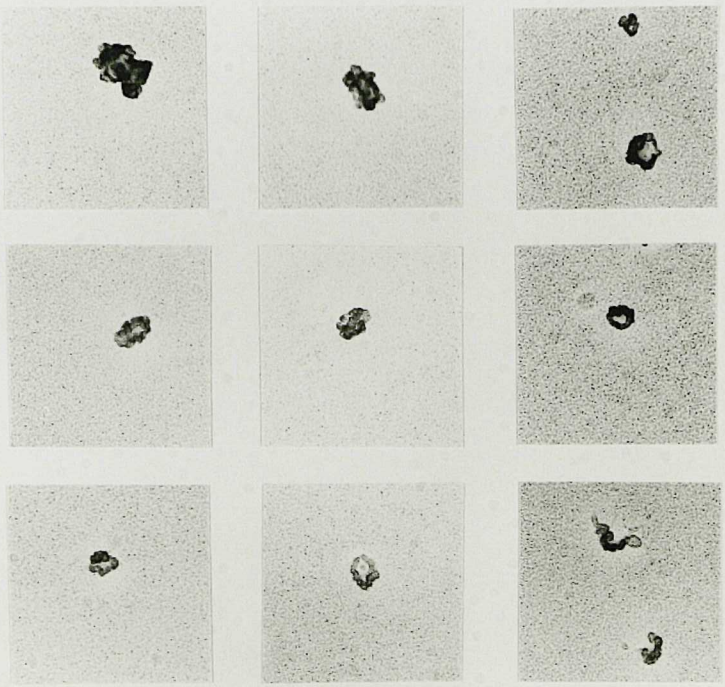
100 nm

VZV Virions Etched 30 Seconds

Figure 29. An electronmicrograph of virions following 40 sec. of etching. There was still a wide range of particle sizes, but some of the particles were in the size range of the core.

VZV Virions Etched 40 Seconds

Figure 30. Selected particles following 40 sec. of etching, at a higher magnification. The bottom 2 rows are particles which may represent cores. At this point they still probably had some capsid proteins attached. Also note the 2 particles in the lower right photo which appeared to be open toroids.



100 nm

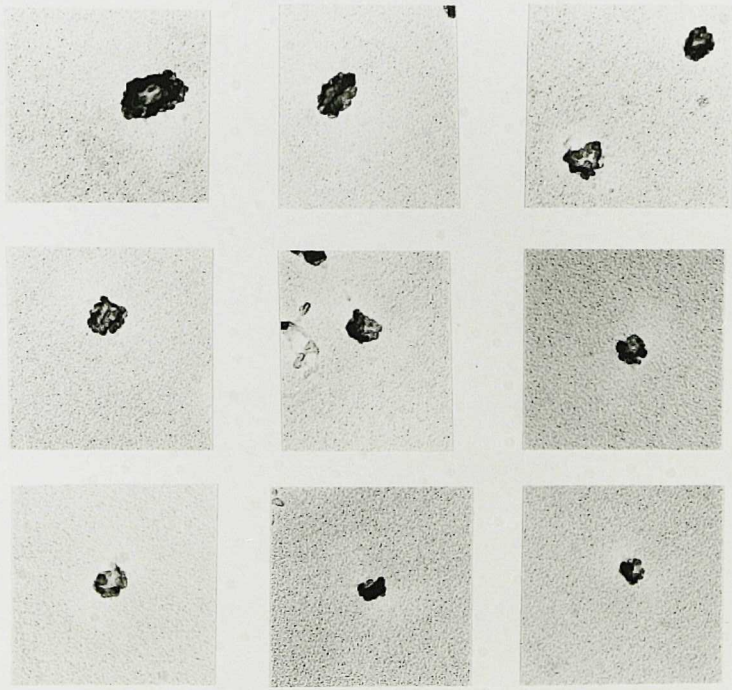
VZV Virions Etched 40 Seconds

Figure 31. An electronmicrograph of virions following 50 sec. of etching. The majority of the particles were the size of the core.

300 nm

VZV Virions Etched 50 Seconds

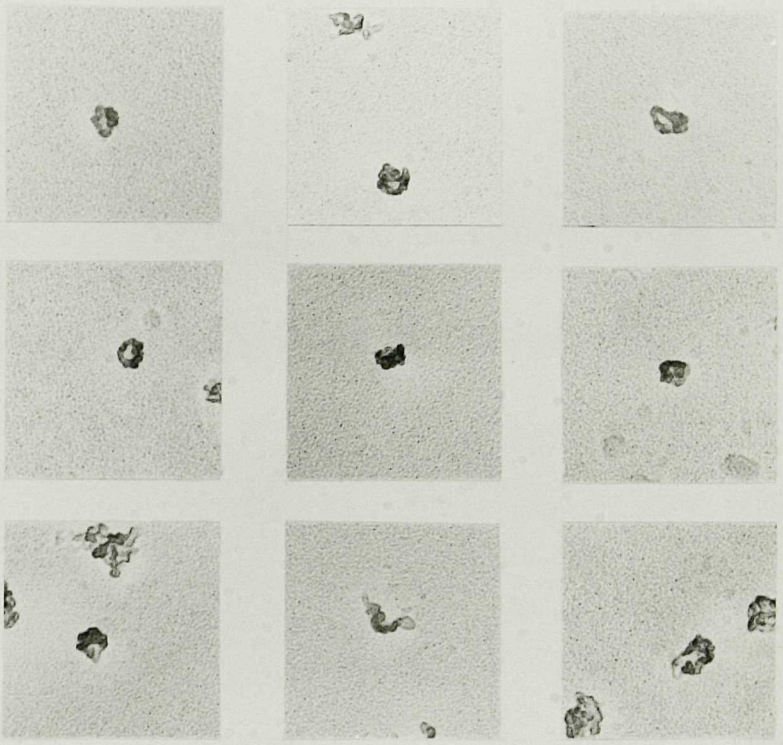
Figure 32. Selected particles, following 50 sec. of etching, at a higher magnification. All but the first 2 had been etched away to where they had a size and shape similar to the toroidal cores seen in thin sections of VZV infected cells.



100 nm

VZV Virions Etched 50 Seconds

Figure 33. Particles seen following 30-50 sec. of etching which appeared to be viral cores.



VZV Cores

remnants of the tegument their normal (knobby) surface structure was obscured; this also may have been the inside of the outer capsid or the outside of the inner icosahedron. Eventually, when all capsid morphology had disappeared, we saw many examples of doughnut shaped structures, both filled and empty, as well as "comma-shaped" structures which may have been doughnuts which had been cut at one point in the circle. These data lend support to the models of VZV core structure which suggest a "toroidal" organization of the DNA (Furlong *et al.*, 1972; Perdue *et al.*, 1976).

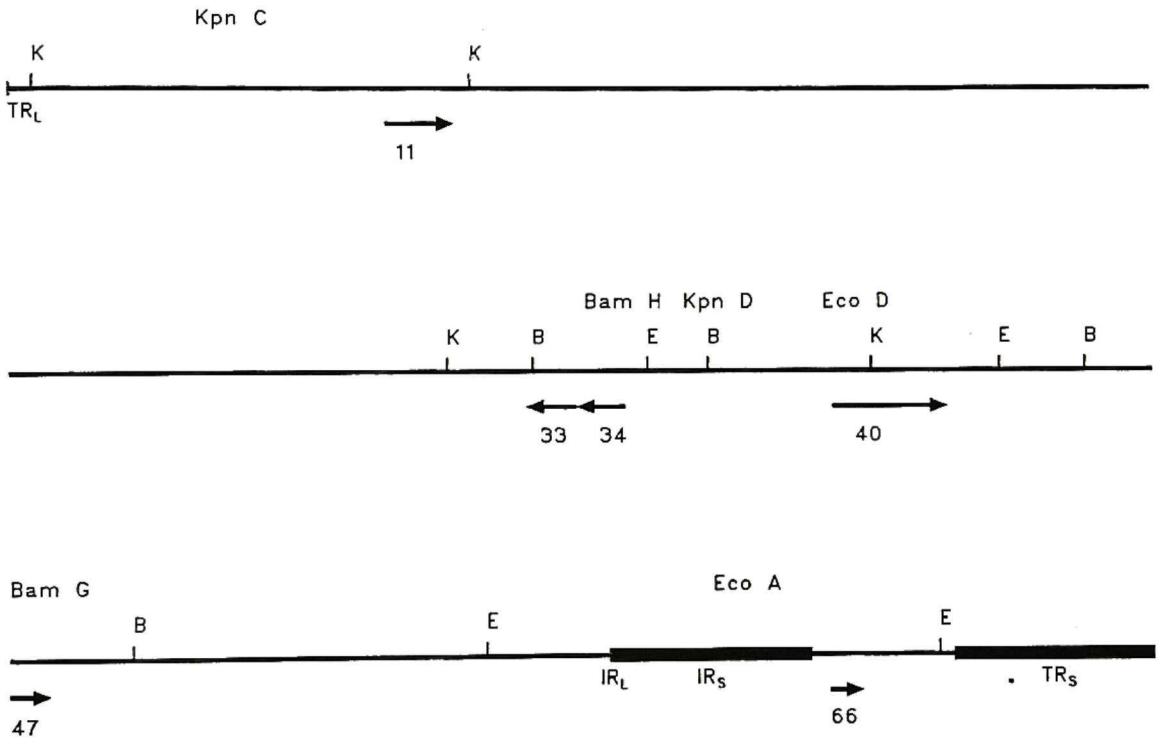
Molecular Cloning of VZV Structural Genes

Except for the glycoproteins, virtually nothing is known about the genetic location of any of the genes for VZV structural proteins. We have shown that the structure of VZV encompasses about 35 proteins, each of which potentially is encoded in one of the VZV genes. The nucleotide sequence of VZV DNA is known, as we have already pointed out, but most of the structural proteins have not been mapped to the 67 open reading frames which have been defined by the sequence. Nevertheless, we have been able to utilize the fact that the genomes of VZV and of HSV are to a large extent colinear, and we have worked out potential coding locations for certain VZV structural proteins based on knowledge of their HSV counterparts.

From the multitude of VZV structural proteins we selected a small number of particular interest to us (figure 34). In addition to correlating particular proteins with a VZV gene, we also hoped to be able to use the cloned genes to investigate the behavior of the expressed protein and, perhaps, examine possible protein-protein interactions leading to capsid assembly *in vitro*. The following strategy was adopted:

Figure 34. A map of the VZV genome showing the location of the ORFs cloned into pSC11 and the fragments, which were in the library of VZV genes we had in our lab, from which they came. Gene 11 was cloned from the Kpn C fragment, gene 33 from the Kpn D fragment, gene 34 from the Bam H fragment, gene 40 from the Eco D fragment, gene 47 from the Bam G fragment, and gene 66 from the Eco A fragment.

VZV GENOME


Kpn C
K
K
TR$_L$
11
Bam H Kpn D Eco D
K B E B K E B
33 34
40
Bam G
Eco A
B
E
E
IR$_L$
IR$_S$
TR$_S$
47
66

an open reading frame of interest was selected, cloned out so that the 5' end of the cloned fragment was close to the ATG start codon and the 3' end close to the translational stop codon. This was then inserted into pSC11, the vaccinia shuttle plasmid, before recombination of the pSC11 derivative with wild type vaccinia virus. The resulting recombinant vaccinia virus, hopefully, would express the VZV protein of interest. The following VZV genes were used:

VZV Gene 40. The major capsid protein of HSV was mapped to 0.23-0.27 on the HSV genome (Costa *et al.*, 1984), and has since been attributed to HSV gene U_L19 (McGeoch *et al.*, 1988). The equivalent VZV gene is ORF40, which encodes a potential protein of 154,971 kDa, with 52% amino acid homology to the HSV protein. The major capsid protein of VZV is about 155,000 kDa on PAGE (Hay *et al.*, 1987) and seems thus very likely to be the product of VZV ORF40. We were especially interested in proving that this was so, since the expressed protein might be capable of some self-association in the context of capsid formation. Unfortunately, cloning of ORF40 turned out to be very awkward, partly because of its size. The vaccinia cloning of ORF40 started with the EcoD fragment; this was cut with Bst X1 to give a 4795 bp fragment. This had four ATG codons upstream from the actual translational start site and, thus, needed to be modified before cloning into vaccinia. It was inserted into the Sma 1 site of pSC11 to give pVZ40BSC (appendix, figure 60). Removal of the upstream ATGs involved a two-step procedure. First, the 275 bp Hpa II/Xba I fragment of EcoD was inserted into pBS, which had been cut with Acc I and Xba I, to give pHX4. A Hpa II fragment may be inserted into an Acc I site with loss of the restriction site. Next, the 4906 bp Xba I fragment of

EcoD was inserted into the Xba I site of pHX4 to give pHX4', which was then cut with Hind III and Eco RI to give a 5131 bp fragment that was cloned into pSC11 to give pVZ40HXSC (appendix, figure 63). The multiple cloning site of pBS had an Sph I site, which contained an ATG, upstream from its Acc I site. Thus, pVZ40HXSC had an extra start site upstream from the authentic start; to remove this, the 2163 bp Pst I fragment of pHX4' was cloned into the Pst I site of pUC9 to give p9VZ40P. The 3751 bp Nae I/Eco RI fragment of pHX4' was then put into p9VZ40P, which had been cut with Nae I and Eco RI, to give p9VZ40. Finally, the 5110 bp Hind III/Eco RI fragment of p9VZ40 was inserted into the Sma I site of pSC11 to give pVZ40SC (appendix, figure 66).

VZV Gene 11. In previous work from our laboratory, the VZV gene 10 protein had been mapped on the VZV genome using antiserum derived from a synthetic peptide made to predicted ORF sequences (Kinchington, P.R., unpublished observations). In HSV, the equivalent to the VZV gene 10 protein is a transactivating factor (αTIF) found in the tegument of the virus; recently it was found that the gene next to HSV αTIF interacts with it to down regulate its activity (McKnight *et al.*, 1987). The VZV homologue of this gene is gene 11; we were thus interested in it both as a tegument protein and as a potential transcriptionally-active protein. The vaccinia cloning of gene 11 was quite straightforward: the KpnC fragment of VZV was cut with Bsp1286 and Mlu I to give a 2769 bp fragment, which codes for an expected protein of 91,825 kDa, which was inserted into pSC11 (appendix, figure 55).

VZV Gene 33. VZV gene 33 is the homologue of HSV gene U_L26, sharing 34% homology at the amino acid level. The U_L26 protein, p40, is

a most interesting molecule in that p40 temperature-sensitive mutants fail to package HSV DNA, but make large numbers of (empty) capsids (Preston *et al.*, 1983). This protein and, by analogy, the VZV gene 33 protein, are strong candidates for a herpesvirus core protein essential for genome packaging. The vaccinia cloning of VZV gene 33 was initiated as follows: the VZV KpnD fragment was cut with Ban I and Dde I to give a 2985 bp fragment containing the gene 33 ORF (66,043 kDa). This was first inserted into the Sma I site of pUC9 to give p9VZ33, which was then cut with Hind III and Eco RI before insertion into the Sma I site of pSC11 to give pVZ33SC (appendix, figure 57).

VZV Gene 34. The HSV homologue to VZV gene 34 (44% homology) is U_L25; its product is a protein defined by the temperature-sensitive mutant 17 *ts* 1204, which has extremely interesting properties. At the non-permissive temperature, no fusion of virions with the cell membrane occurs and, if the virus is allowed to enter cells at the permissive temperature, upon temperature shift-up no capsids are assembled (Addison *et al.*, 1984). Thus this is a single genetic lesion with more than one phenotypic effect; we were interested in expressing the equivalent VZV protein of 65,182 kDa. The cloning of this ORF into vaccinia was quite straightforward: VZV BamH was cut with Cla I and Stu I to give a 2052 bp fragment which was inserted into the Sma I site of pSC11 to give pVZ34SC (appendix, figure 59).

VZV Gene 47. The HSV homologue to VZV gene 47 (55% homology) is U_L13 which has been identified as containing a protein kinase domain (Smith and Smith, 1989). The cloning of this gene was also quite straightforward. The BamG clone 1,867 Hga I fragment contains the whole gene. This fragment was blunt ended and cloned directly into the Sma I site of pSC11

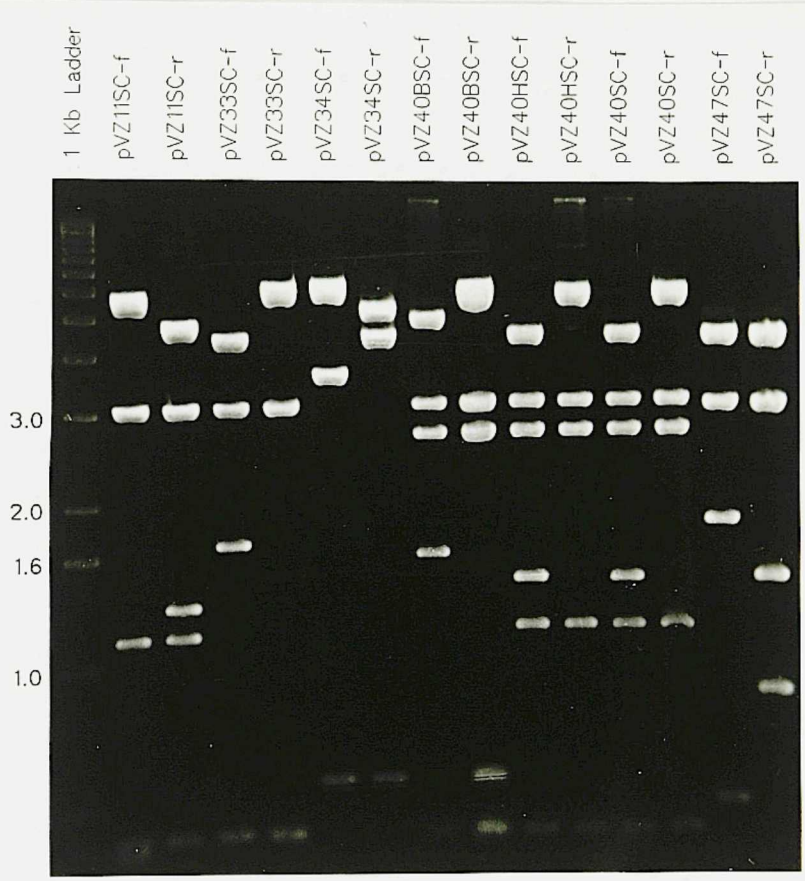
(Appendix, figure 68).

All pSC11 derivatives were then recombined into vaccinia virus using infection with vaccinia strain wr and transfection with each of the pSC11 clones. Following homologous recombination between the TK gene of the vaccinia genome and the vaccinia TK fragments in pSC11, vaccinia recombinants were selected which were TK $^-$, expressed β-galactosidase and carried the VZV genes.

To insure that they were in the proper orientation downstream from the p7.5 promoter the pSC11 clones were cut with specific restriction enzymes, and their fragment sizes compared with those given from a computer analysis of their sequences by the IBI DNA analysis program. Figure 35 is an agarose gel of the clones for the VZV genes 11, 33, 34, 40, and 47 showing each gene in both orientations in the plasmid. All of the clones were cut with Bam HI except for the gene 34 and 47 clones which were cut with Pst I and Eco RI respectively. The difference in the gene 40 Bst XI clone and the other 2 gene 40 clones was because the others were first cloned into pUC9 and picked up an extra Bam HI site when they were cloned from there into pSC11.

As a check on the structure of the vaccinia recombinants, the following procedure was used: the Sma I cloning site in pSC11 was sandwiched between a Bam HI site and an Eco RI site. This allowed the gene of interest to be cut out of the pSC11 clone or the genome of the vaccinia recombinants using these two restriction enzymes. DNA isolated from each of the recombinants and vaccinia wr virus was isolated and cut with Eco RI and Bam HI along with their respective pSC11 clones. The digests were run on an agarose gel and southern blotted on Biodyne mem

Figure 35. A photograph of a 1% (w/v) agarose gel, stained with ethidium bromide, of the pSC11 clones constructed from VZV genes 11, 33, 34, 40, and 47, showing the genes in both forward (f) and reverse (r) orientations in the plasmid. Genes 11, 33, and 40 were cut with Bam HI, gene 34 was cut with Pst I, and gene 47 was cut with Eco RI. Size, in Kb, is listed on the left for some of the marker bands.



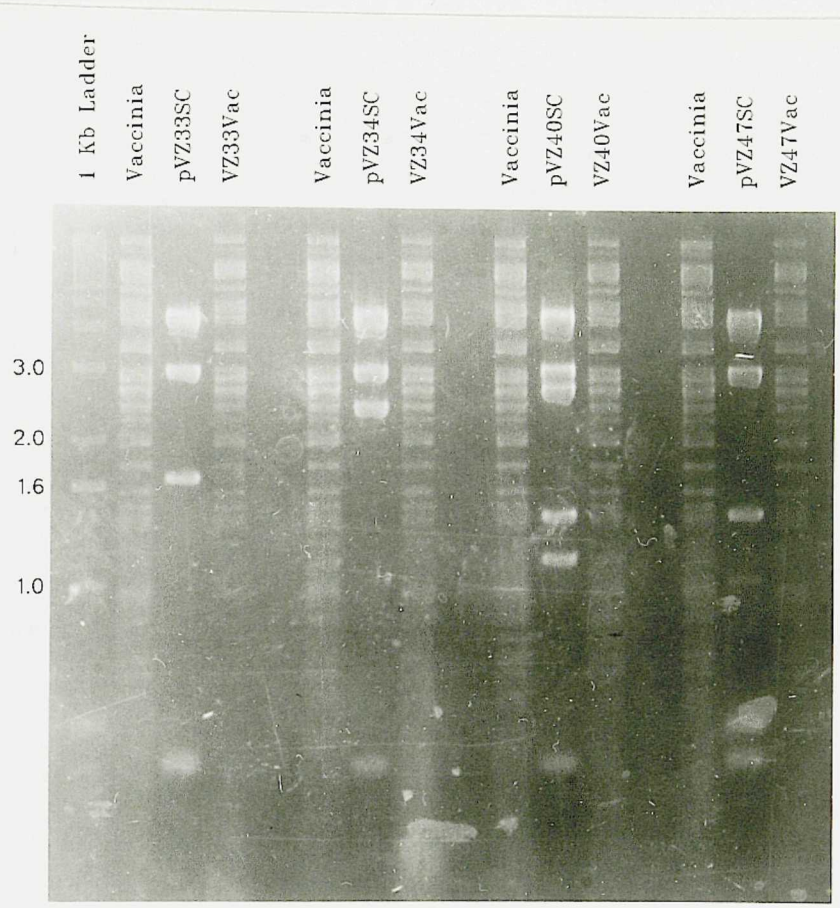
pSC11 Clones

branes. Each clone was probed with random primer labelled VZV DNA isolated from the pSC11 clones. Equivalent amounts of vaccinia, recombinant, and plasmid DNA were run in each lane, but since the size of the plasmid was much smaller than the vaccinia genome, more copies of the VZV genes were seen in the plasmid lanes (figure 36). The bands present in the recombinant lanes were the expected sizes for each clone, and had corresponding bands in their pSC11 precursors (figures 37 and 38). The extra bands in the pSC11 lanes were probably due to pSC11 DNA which co-migrated with the VZV DNA and was isolated along with the VZV DNA used to make the probes. The sizes of the bands expected from the clones were calculated from the known sequences of the clones and are indicated on the photographs. As far as we can tell from these analyses, both the sizes and orientations of the VZV inserts in both pSC11 and in the vaccinia recombinants were correct and we expected that the VZV ORFs would be expressed in the corresponding vaccinia chimeras.

The vaccinia clones were used to inoculate rabbits in an attempt to make antibodies against the cloned genes, since otherwise we have no means of assessing their ability to produce VZV proteins. For example, not unexpectedly, there were no indications of new protein bands on silver stained gels with the vaccinia recombinants. Rabbits were injected intradermally with the live virus on a bi-weekly schedule, with a test bleed being taken at the time of each injection. The rabbits produced antibodies against many of the vaccinia proteins (figure 39) but have not yet produced antibodies to any of the VZV gene products.

As a control for these experiments testing rabbit sera for anti-VZV protein antibodies, we set up western blots of uninfected cells, VZV-

Figure 36. A photograph of a 1% (w/v) agarose gel, stained with ethidium bromide, of the DNAs isolated from the vaccinia recombinants of VZV genes 33, 34, 40, and 47, prior to being blotted to Biodyne for southern blots. The DNA from each recombinant was run alongside the pSC11 clone from which it was constructed and vaccinia virus DNA. All of the DNAs were cut with Bam HI and Eco RI. Size, in Kb, is listed on the left for some of the bands of the marker lane.



Vaccinia Recombinants

Figure 37. Autoradiographs of southern blots of the vaccinia recombinants, with VZV genes 33 and 34, run alongside the pSC11 clones from which they were constructed and vaccinia DNA. The DNAs were cut with Bam HI and Eco RI and separated on a 1% agarose gel (shown in fig. 36), blotted to Biodyne, and probed with DNA, specific for each gene, which had been cut from their respective pSC11 clones and isolated from agarose gels. The probe DNA was labelled with α^{32}P-CTP by random primer labelling. The size markers alongside each blot are the expected sizes (kDa) of the VZV genes from each of the recombinants. Extra bands seen in the pSC11 lanes are due to contamination of the probe DNA with pSC11 DNA.

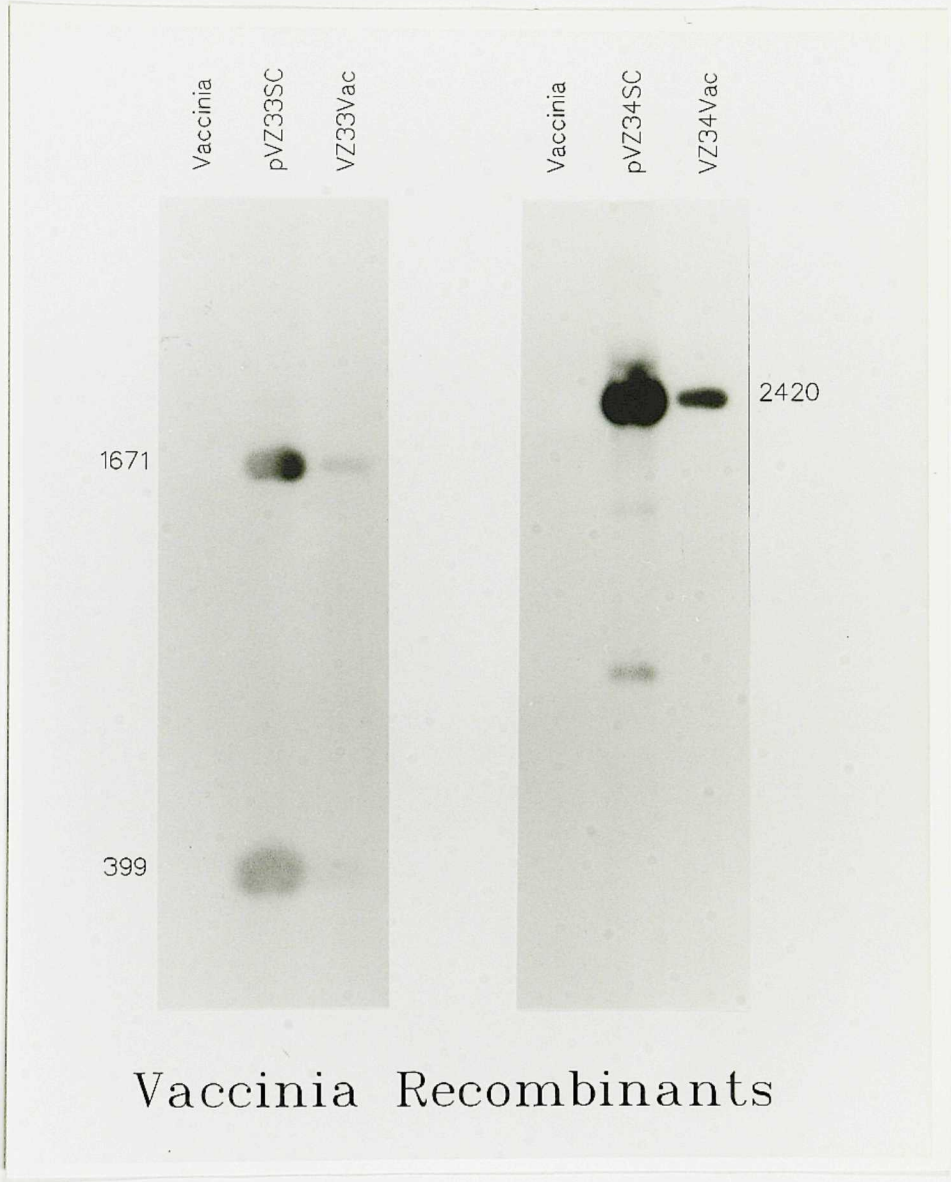
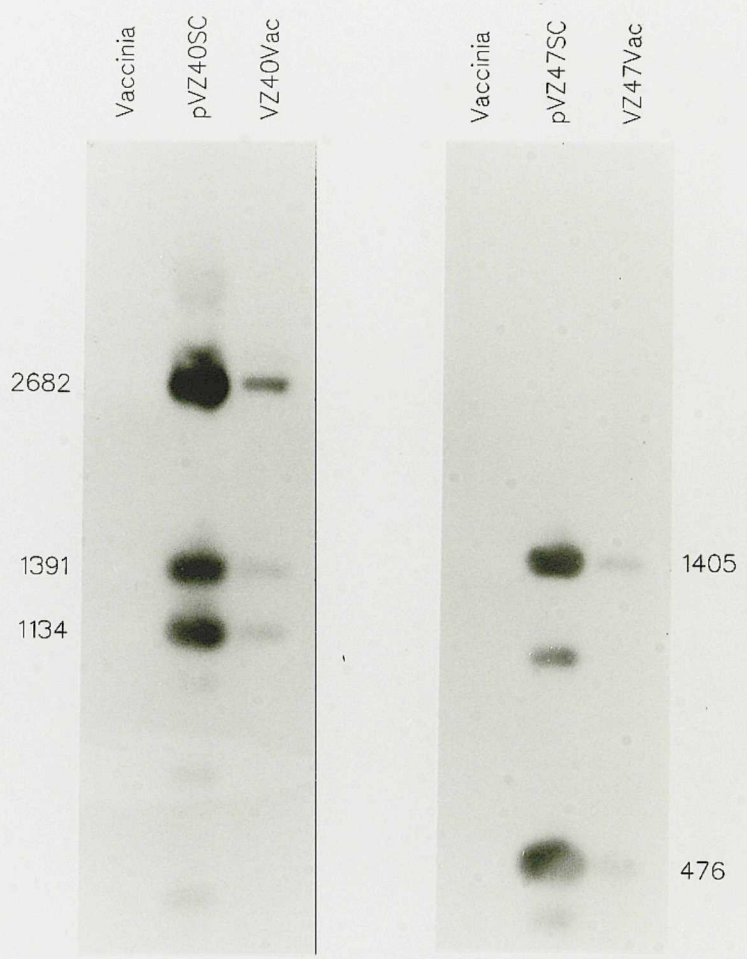
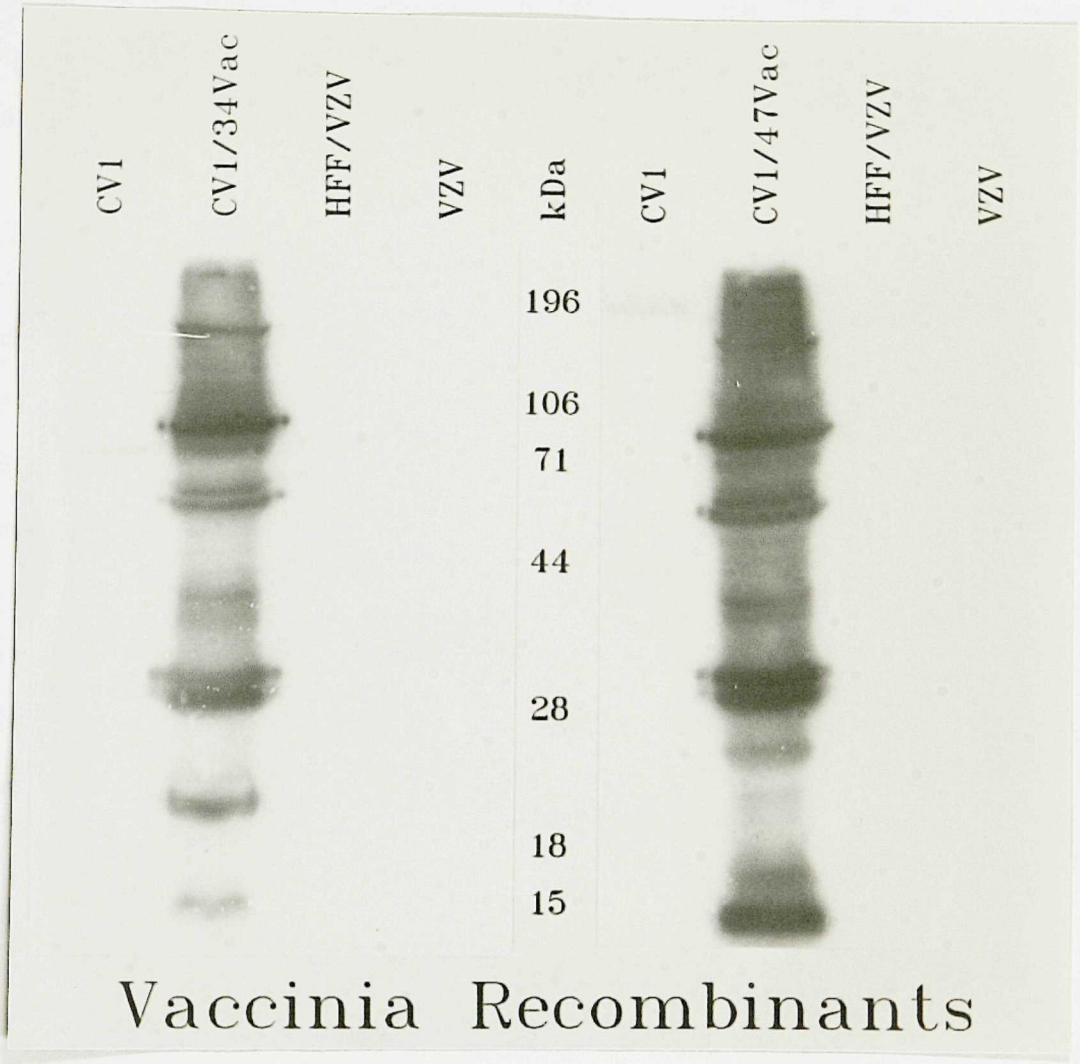

Vaccinia
pVZ33SC
VZ33Vac
Vaccinia
pVZ34SC
VZ34Vac
2420
1671
399
Vaccinia Recombinants

Figure 38. Autoradiographs of southern blots of the vaccinia recombinants containing VZV genes 40 and 47. The blots were done the same as described for the blots in the previous figure. Extra bands in the pSC11 lanes are due to contamination of the probe DNA with pSC11 DNA.



Vaccinia Recombinants

Figure 39. Autoradiographs of western blots of proteins from CV1 cells, CV1 cells infected with the VZV/vaccinia recombinants 34Vac and 47Vac, HFF cells infected with VZV, and purified VZ virions, reacted with antisera from rabbits inoculated with the recombinants. Positions of the molecular weight markers are shown between the two autoradiographs.



Vaccinia Recombinants

infected cells and purified VZV virions, reacting them with sera for two known VZV proteins, a late structural protein, ORF10, the HSV αTIF equivalent, and an early non-structural protein, ORF29, the HSV ICP8 equivalent. As can be seen in figure 18, the structural protein ORF10 is present in infected cells and in virions and the non-structural ORF29 is only present in infected cells. Thus, if our rabbits were producing antibodies to VZV proteins we should expect to detect them.

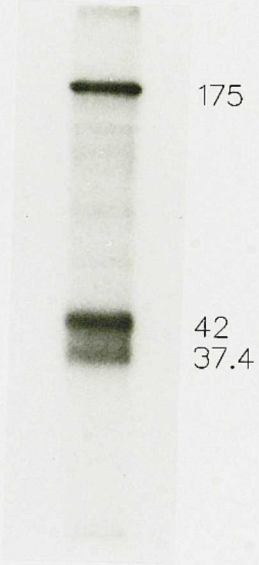
VZV-Associated Protein Kinase Activities

Among the most interesting proteins associated with viruses and with virus-infected cells are the protein kinases. They have, as pointed out in the introduction to this dissertation, been associated with modification of proteins which might play direct or indirect roles in the control of cellular metabolism and/or the control of virus replication.

Recent studies with herpes simplex virus have shown that purified virions are associated with protein kinase activity (Leader and Katan, 1988) and earlier work from our laboratory (Roberts *et al.*, 1985) had shown that several phosphoproteins were present in VZV-infected cells and the virions. With this as background, we decided to explore the kinase activity of purified VZV virions.

VZV was purified using the sucrose gradient/tartrate gradient procedure described earlier in the materials and methods section of this work. When these purified VZV virions were put into a buffer system containing a small amount of NP40, which disrupted the viral membrane, along with γ^{32}P-ATP, protein kinase activity was readily detected. Several virion proteins became labelled, with three major labelled proteins appearing at 175, 42 and 37.4 kDa (figure 40). In a series of experi

Figure 40. An autoradiograph showing the results of the kinase activity associated with purified VZV virions. Following the kinase reaction the virion proteins were separated on a 10% (w/v) SDS-PAGE gel which was dried and exposed to x-ray film. The molecular weights (kDa) of the 3 major bands phosphorylated are shown.



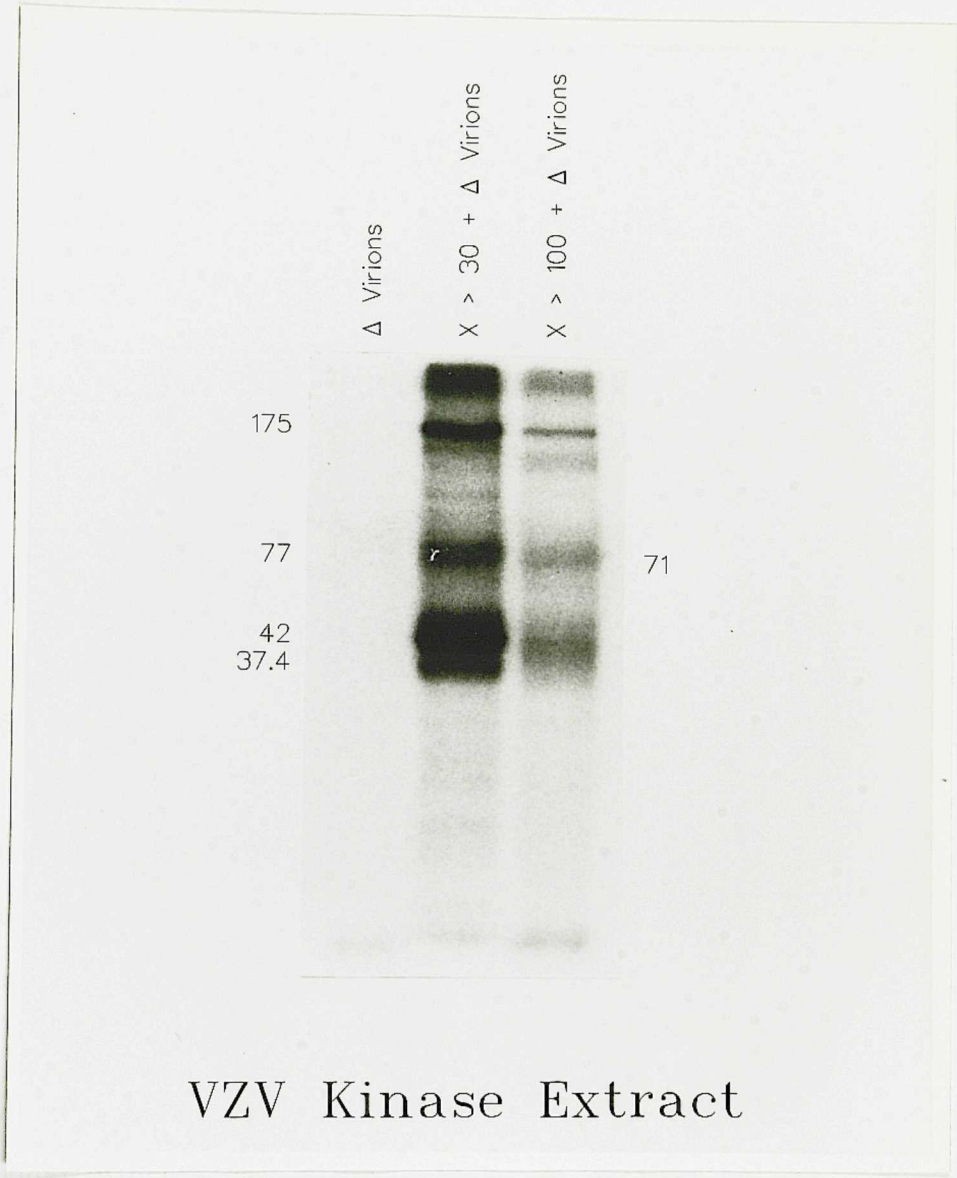
VZV Protein Kinase Activity

ments, the optimum concentrations for NaCl, Mg^{++}, and ATP in the buffer were worked out and incorporated in all subsequent experiments. In a further series of experiments, we were able to demonstrate that the VZV virion kinase activity was destroyed by heating the virions to 68°C for 15 mins.

This protein kinase activity was extracted from purified virions using 10% (v/v) NP40 and 0.6 M NaCl. In order to size the kinase activity and to get rid of the salt and detergent in the extract, two types of filters, Centricon 30 and Centricon 100 Amicon filters, were used to treat the extract. The retentates from treatment with each of these filters was added to heat-inactivated virions to assess their ability to label the proteins labelled by the virions themselves (figure 41). Each fraction appears to give a different labelling pattern of the viral proteins. The retentate from the Centricon 100 filter does not label the 37 kDA protein. This suggests that there are two kinases present in the virions. Both are retained by the Centricon 30 filter, but only one is retained by the Centricon 100 filter. There are also new bands present at approximately 71 and 77 kDA which we feel are probably breakdown products of the 175 kDa protein, found in the extract.

In experiments described below, we have cloned and expressed the proteins corresponding to two putative protein kinase activities encoded by VZV, in gene 47 and gene 66. We have attempted to test these proteins for kinase activity by adding *in vitro* translation products of VZV genes 47 and 66 to heat inactivated virions and assaying for enzyme activity. Disappointingly, it was found in these experiments that the rabbit reticulocyte lysate (used for the translation) had an inhibitory effect

Figure 41. An autoradiograph of the VZV proteins labelled when the C-30 and C-100 retentates of the soluble virion kinase extract were added to heat inactivated virions in the kinase reaction. The labelled proteins were run alongside heat inactivated virions, also put into the kinase reaction, on a 10% (w/v) SDS-PAGE gel. The molecular weights (kDa) of the major bands are listed alongside them.



VZV Kinase Extract

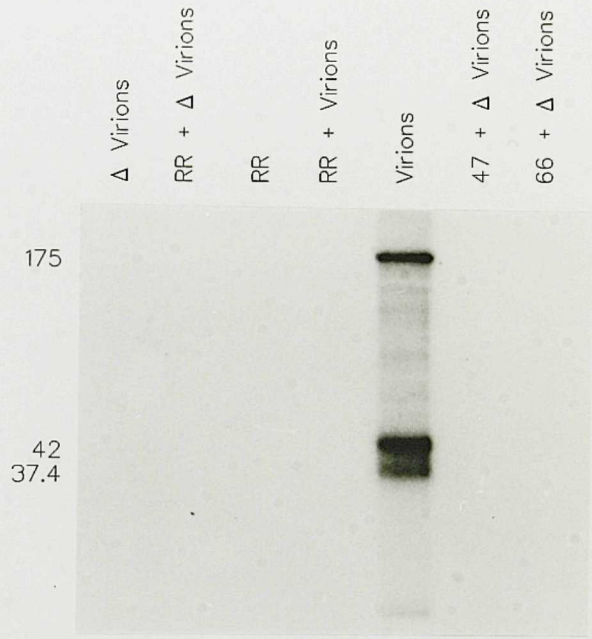
upon the kinase reaction (figure 42); the control containing non heat-inactivated virions and the lysate alone in the kinase reaction buffer showed no activity. Neither dialysis of the lysate nor chromatography on a spin (Sephadex G25) column abolished the kinase inhibitor (figure 43), but a series of dilutions of the lysate, in PBS, added to the reaction showed that the inhibitor could be gotten rid of (figure 44).

An attempt was next made to remove the lysate inhibitory factor by using Amicon filters. This was achieved, and the retentate from diluted lysate spun through an Amicon 30 filter no longer was inhibitory for the virion associated kinase, as we had hoped. Unfortunately, this treatment also allowed the kinases present in the lysate itself to become active. But, if this retentate was put through an Amicon 100 filter the lysate kinases were also removed (figure 45).

It is interesting to note that two kinases have been identified in rabbit reticulocyte lysate. One is activated by dsRNA and the other is inhibited by the presence of heme (Farrell *et al.*, 1977). Since no dsRNA is present in the virions the active lysate kinase must be the heme inhibited kinase, and whatever factor is inhibiting the virion kinases is also removed along with the heme.

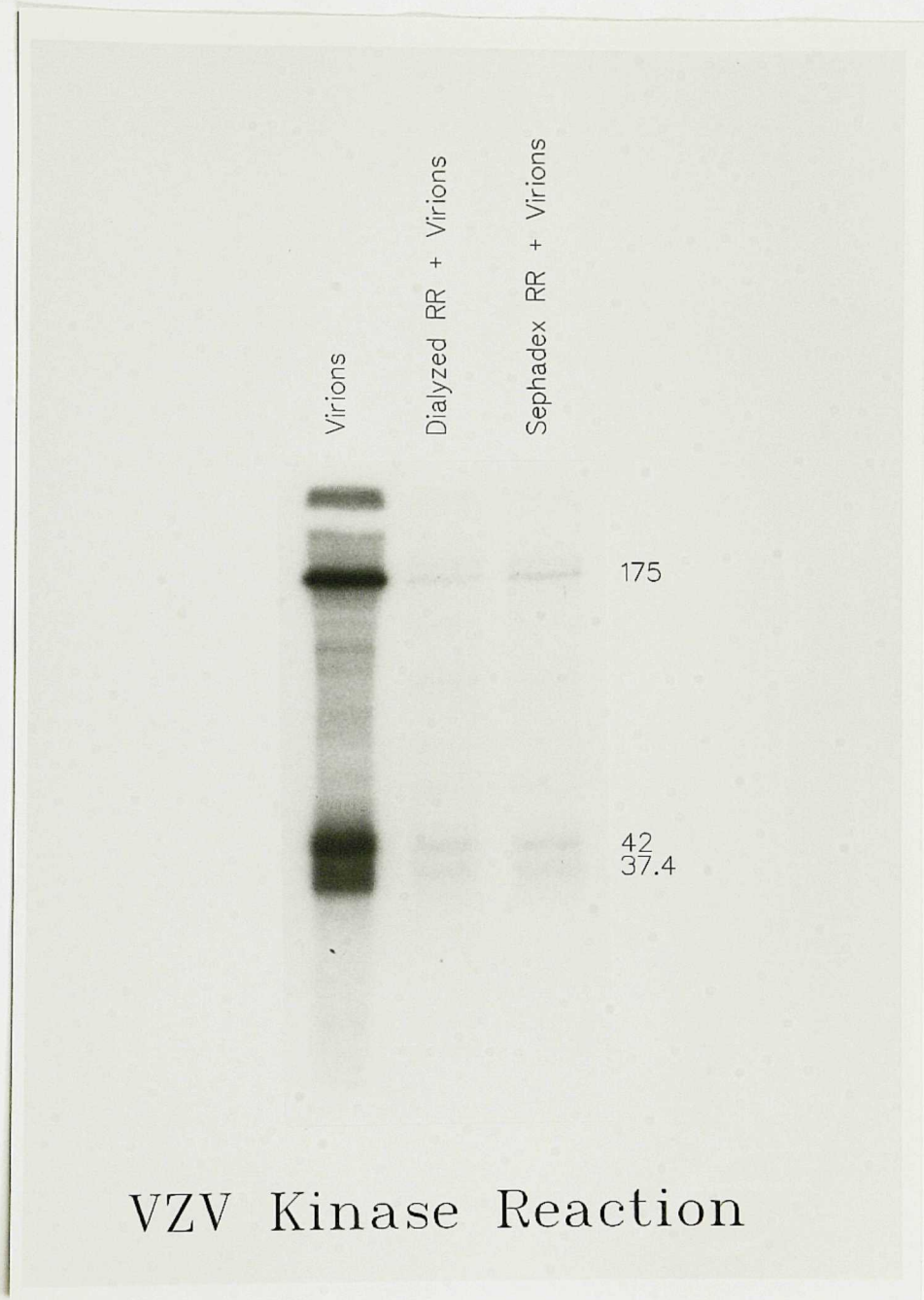
At this stage, we had identified VZV virion protein kinase activity(ies), but were finding them awkward to isolate and study by conventional biochemical means. Thus, we decided to use a different approach, recently developed, of direct assay for kinase activity on a protein blot. When cell extracts are run on an SDS-PAGE gel and the proteins transferred to an Immobilon membrane, polypeptides can be renatured and tested for autophosphorylation (a common property of protein

Figure 42. An autoradiograph of the SDS-PAGE gel showing the results of an experiment adding the *in vitro* translation products of the Bluescript clones of VZV genes 47 and 66 to the *in vitro* VZV kinase reaction along with heat inactivated virions. The following were added to the reaction mix: lane 1, heat inactivated VZV; lane 2, rabbit reticulocyte lysate and heat inactivated VZV; lane 3, rabbit reticulocyte; lane 4, rabbit reticulocyte and VZV; lane 5, VZV; lane 6, the gene 47 translation mix and heat inactivated VZV; lane 7, the gene 66 translation mix and heat inactivated VZV. The molecular weights (kDa) of the major bands are listed alongside the gel.



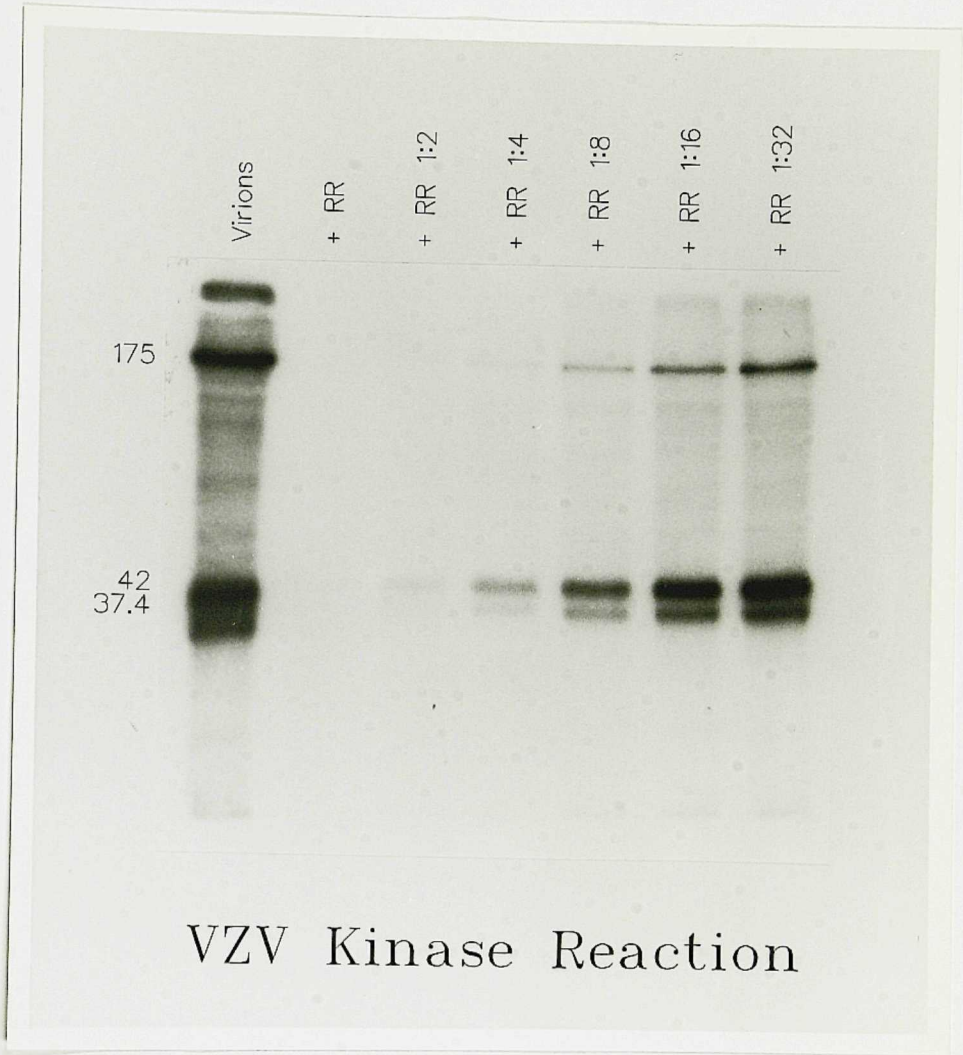
pVZ47BS and pVZ66BS Translation Products
Kinase Activity

Figure 43. An autoradiograph of an SDS-PAGE gel of rabbit reticulocyte lysate added to the *in vitro* VZV kinase reaction following dialysis or being put through a Sephadex G25 spin column. The molecular weights (kDa) of the major bands are listed alongside the gel.



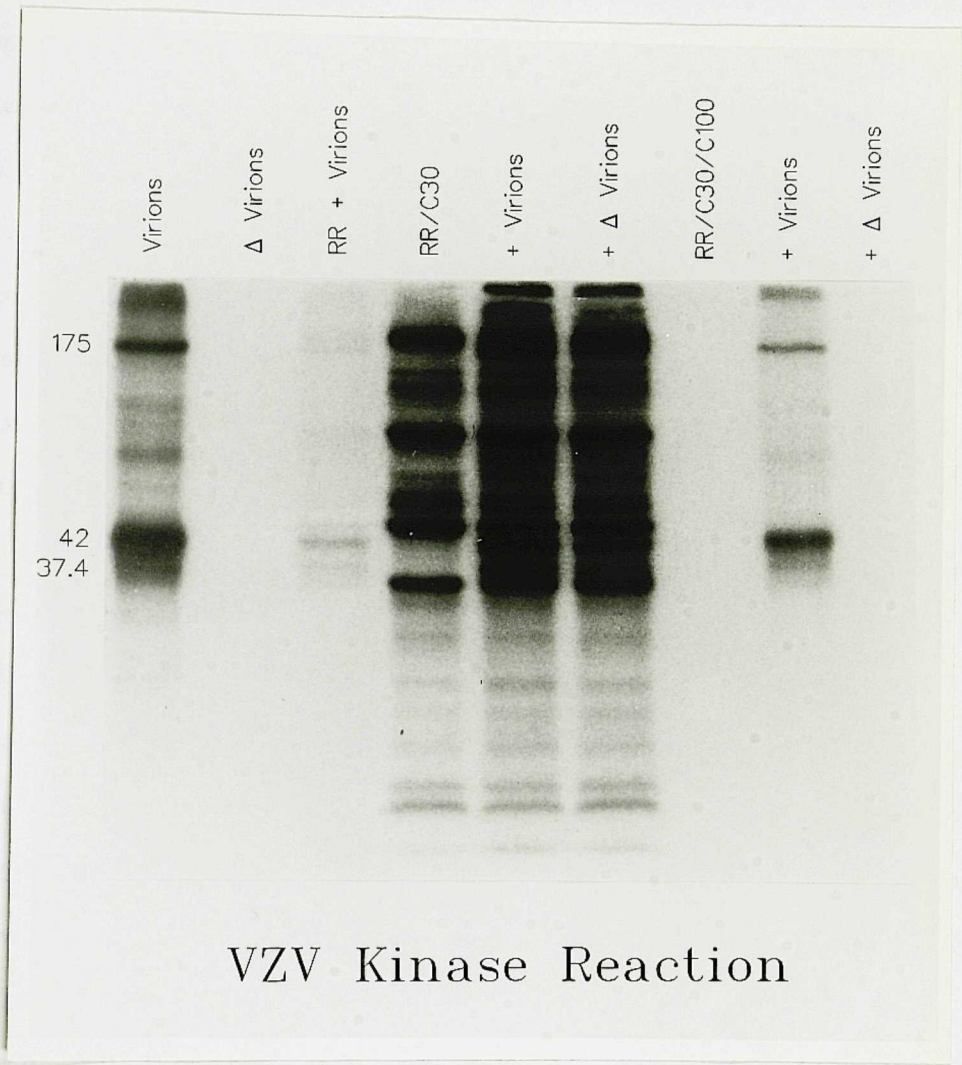
VZV Kinase Reaction

Figure 44. An autoradiograph of the SDS-PAGE gel of a series of dilutions of the rabbit reticulocyte lysate added to the *in vitro* VZV kinase reaction along with VZV virions. The molecular weights (kDa) of the major bands are listed alongside the gel.



VZV Kinase Reaction

Figure 45. An autoradiograph of the SDS-PAGE gel of an experiment showing the effect of filtration with Amicon C-30 and C-100 filtration on the rabbit reticulocyte lysate prior to adding it to the *in vitro* VZV kinase reaction. The following were added to the reaction mix: lane 1, VZV; lane 2, heat inactivated VZV; lane 3, rabbit reticulocyte lysate and VZV; lane 4, the retentate from the C-30 filtration of the lysate; lane 5, the C-30 retentate and virions; lane 6, the C-30 retentate and heat inactivated virions; lane 7, the C-30 retentate of the C-100 filtrate of the rabbit reticulocyte lysate; lane 8, the C-30 retentate of the C-100 filtrate and virions; lane 9, the C-30 retentate of the C-100 filtrate and heat inactivated virions. The molecular weights (kDa) of the major bands are listed alongside the gel.

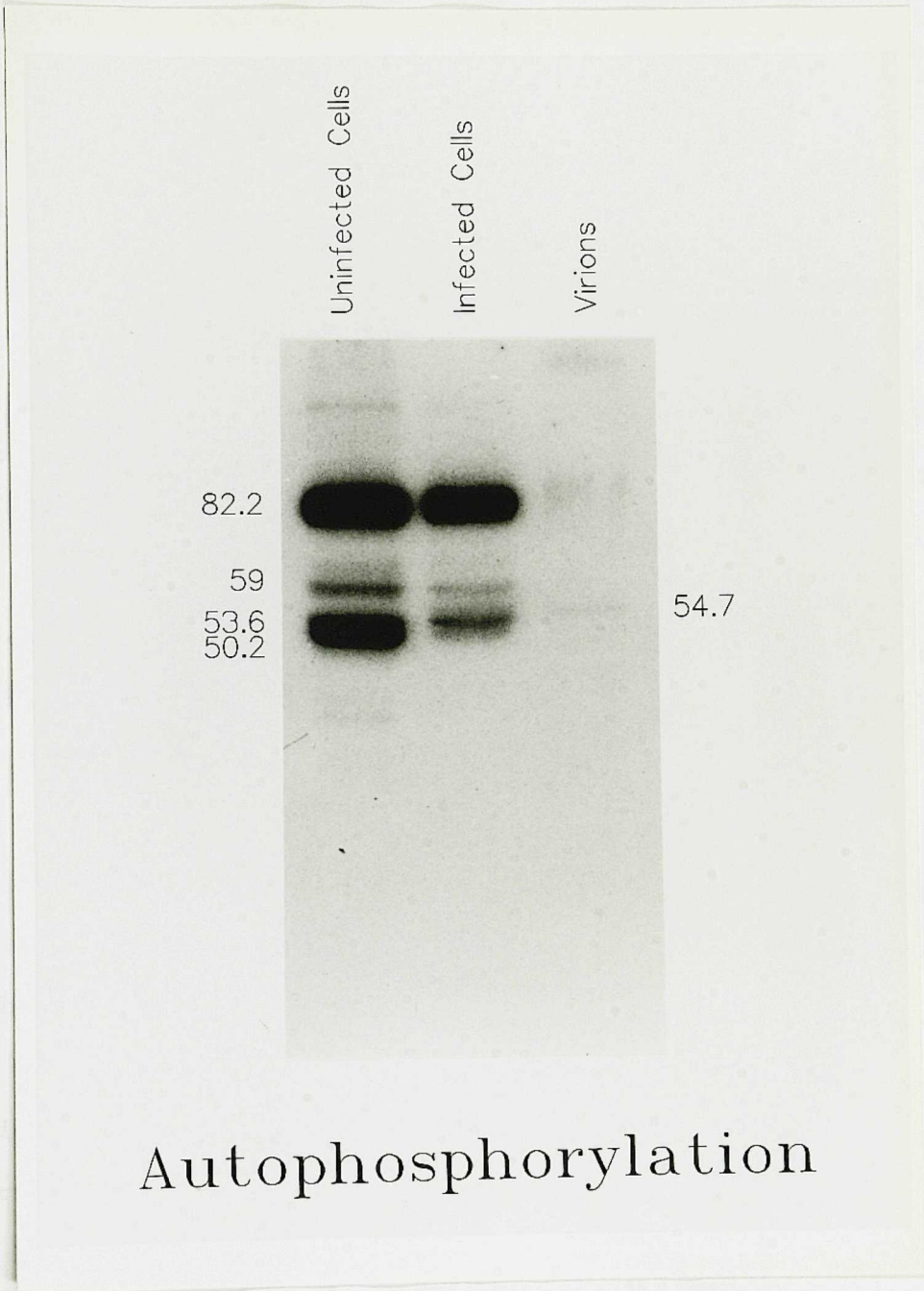


VZV Kinase Reaction

kinases) if the blot is placed in buffer containing γ^{32}P-ATP. This was done with purified VZV virions, VZV-infected cells, and with uninfected cells (figure 46). Seven bands were seen in the uninfected cells, representing the cell's complement of protein kinase activity. However, in VZV-infected cells, several of these bands were markedly decreased and a new band at 54.7 kDa appeared. Interestingly, this new band is also seen in the virion lane along with a faint band at 82 kDa which was probably a cellular kinase, since it was present in large amounts in uninfected cells. Also visible in the virions was a very large mol. wt. band near the top of the gel which we suspect was aggregated material which had trapped some of the kinase activity or bound some of the labelled ATP. This result suggests that there may be both cellular (82 kDa) and viral (55 kDa) protein kinases in the virion.

Having identified three major proteins in VZV virions which are phosphorylated by the virion kinases, we became interested in their identity. All three appear to be previously-recognized phosphoproteins and, probably, DNA-binding proteins (Roberts *et al.*, 1985). We have started by looking at the 175 kDa phosphoprotein. In our laboratory, we have generated an antiserum to the product of the open reading frame (ORF 62), which encodes the likely major immediate early transactivating protein of VZV, IE62, using a synthetic peptide (Kinchington, P.R., Hougland, J., Ruyechan, W.T. and Hay, J., manuscript in preparation). This ORF encodes a polypeptide of about 150,000 which, with phosphorylation or other post-translational modification, might behave as a larger protein on PAGE; thus, we tested the possibility that our virion 175 kDa phosphoprotein might be the ORF 62 product. Uninfected cells, VZV-

Figure 46. An autoradiograph of the autophosphorylated proteins of HFFs, HFFs infected with VZV, and purified VZV. The proteins were separated on a 10% SDS-PAGE gel and blotted to immobilon. The proteins were renatured as described in materials and methods and allowed to autophosphorylate by the addition of γ^{32}P-ATP. The molecular weights (kDa) of the major bands are listed alongside the gel.



Autophosphorylation

infected cells and VZV virions were blotted onto nitrocellulose after PAGE and reacted with the anti ORF 62 antiserum and then with ^{125}I staph A protein; virions were made in the presence and absence of the protease inhibitors TPCK and TLCK. From the autoradiograph (figure 47) it is clear that the ORF 62 product is largely a 175 kDa protein, and that it is present in the VZV virion.

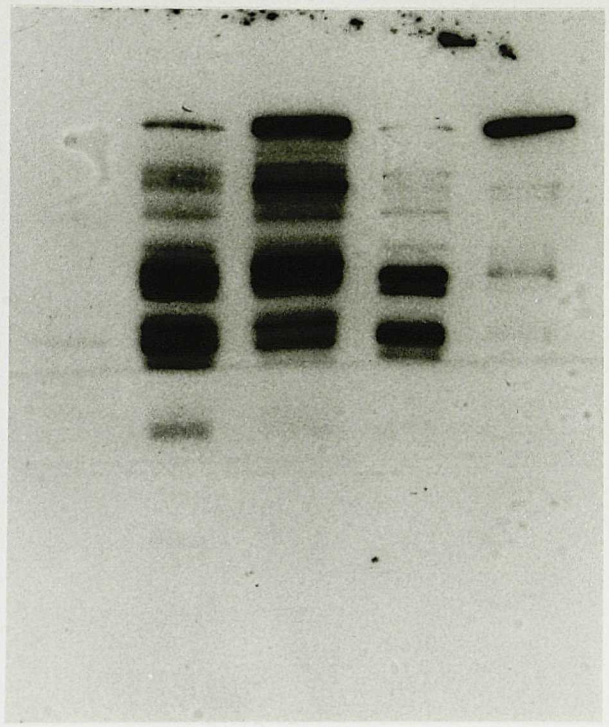
Dr. Kinchington has shown that IE62 is a phosphoprotein by immunoprecipitation of the phosphorylated protein from cells infected with either VZV or a vaccinia recombinant of ORF 62 and incubated with ^{32}P (figure 48). It is, therefore, a very good candidate for the large phosphoprotein seen in our virion kinase experiments; given its probable role in the control of viral gene expression, its phosphorylation may be a significant factor in the ability of VZV to initiate successful infection of the host cell.

VZV-Coded Protein Kinase Activities

We have shown that VZV virions contain protein kinase activity by direct assay of detergent-treated particles. Using a blot procedure, we found that two species, 55 and 82 kDa, are prominent in virion preparations. We were interested in the possibility that one (or both) of these activities might be encoded in the VZV genome and decided to investigate this further.

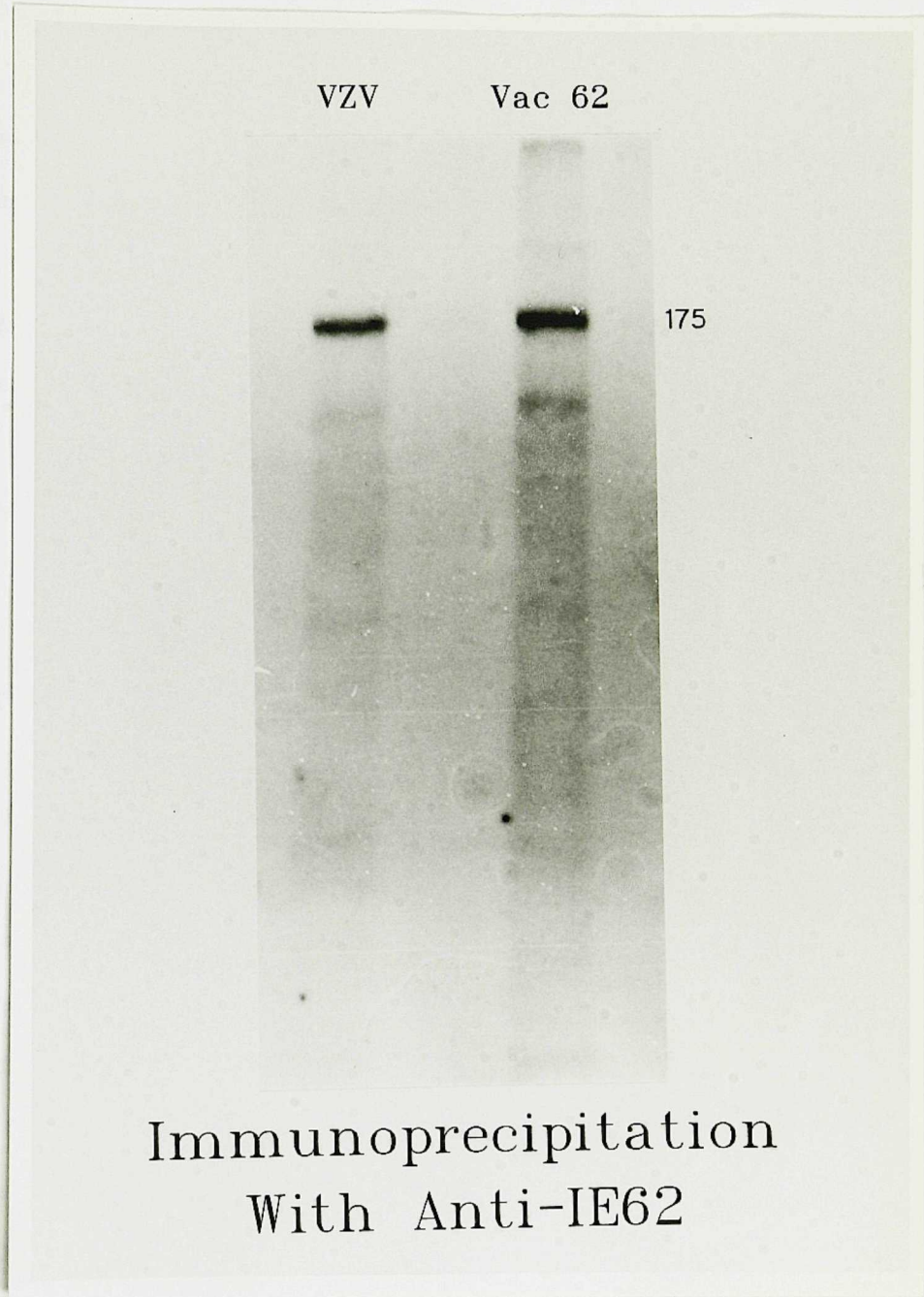
In HSV, a protein kinase activity has been identified as the product of the U_S3 gene (Frame *et al.*, 1987); the VZV homologue of this gene is gene 66. Eukaryotic protein kinases contain specific highly-conserved motifs (Hanks *et al.*, 1988), which can be used to scan genomic

Figure 47. An autoradiograph of a western blot of HFFs, HFFs infected with VZV strain Scott, and purified VZV virions, showing the sensitivity to degradation of the IE62 gene product (the HSV-1 ICP4 equivalent) by cellular proteinases. The virions and infected cells were harvested in the presence (+) or absence (-) of a mixture of the proteinase inhibitors TLCK and TPCK. The blot was probed with rabbit antibody against a synthetic peptide of the carboxy terminus of the protein. The antibody was labelled with ^{125}I labelled staphylococcus protein A. The major 175 kDa protein is indicated at the right.



IE62

Figure 48. Autoradiograph of VZV IE62 immunoprecipitated using rabbit antibody generated against a synthetic peptide from the carboxy-terminus of VZV ORF 62 and run on a 7.5% SDS-PAGE gel. Cells were infected with VZV or a vaccinia recombinant expressing VZV ORF 62 and labelled with ^{32}P orthophosphate. The major 175 kDa protein is indicated at the right. Photograph kindly provided by Dr. Paul Kinchington.



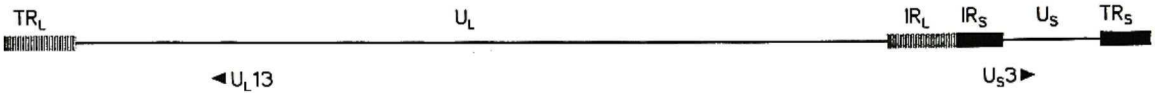
Immunoprecipitation
With Anti-IE62

sequences for putative kinase activities. Recently, Smith and Smith (1989) have scanned herpesvirus genomes and have described two putative protein kinases in VZV, genes 66 and 47 (figure 49). We decided to attempt to express the protein products of these genes and start their characterization. Initially, clones of VZV genes 47 and 66 were made using the pBluescript phagemid (figure 50). This vector has a multiple cloning site between bacteriophage T3 and T7 promoters. A gene can be cloned into this site in either orientation and be transcribed by using the appropriate bacteriophage RNA polymerase. The RNA transcripts may then be used for *in-vitro* translation, using the standard rabbit reticulocyte lysate system.

Figure 51 is an autoradiograph of the *in-vitro* transcripts made from the Bluescript clones by Dr. Michael Pensiero, labelled with ^{32}P-UTP. As can be seen, the transcripts were the expected sizes for mRNAs derived from the two VZV genes. Similar, unlabelled transcripts were then added, by Dr. Pensiero, to a rabbit reticulocyte translation system in the presence of ^{35}S-methionine. The products showed a major 58.6 kDa product from gene 47 and 54.8 and 51 kDa products from gene 66 (figure 52). When these translation products were run alongside virion proteins labelled by the *in vitro* kinase reaction no obvious bands in the virions corresponded in size to the translation products (figure 53), however, if the kinase were present in the virions in minute quantities it might not be detectable by these methods. This is the first description of polypeptide products from either of these genes. The two major translation products for gene 66 may be the result of secondary start site 91 amino acids downstream of the original start site.

Figure 49. A map of the genomes of HSV-1 and VZV showing the locations of the protein kinase genes. VZV gene 47 is homologous to HSV-1 U_L13 and VZV 66 is homologous to HSV-1 U_S3.

HSV 1



VZV

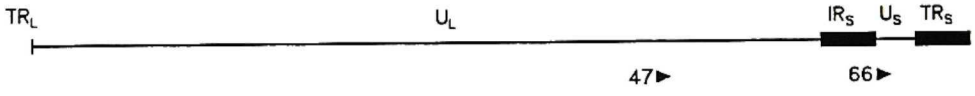
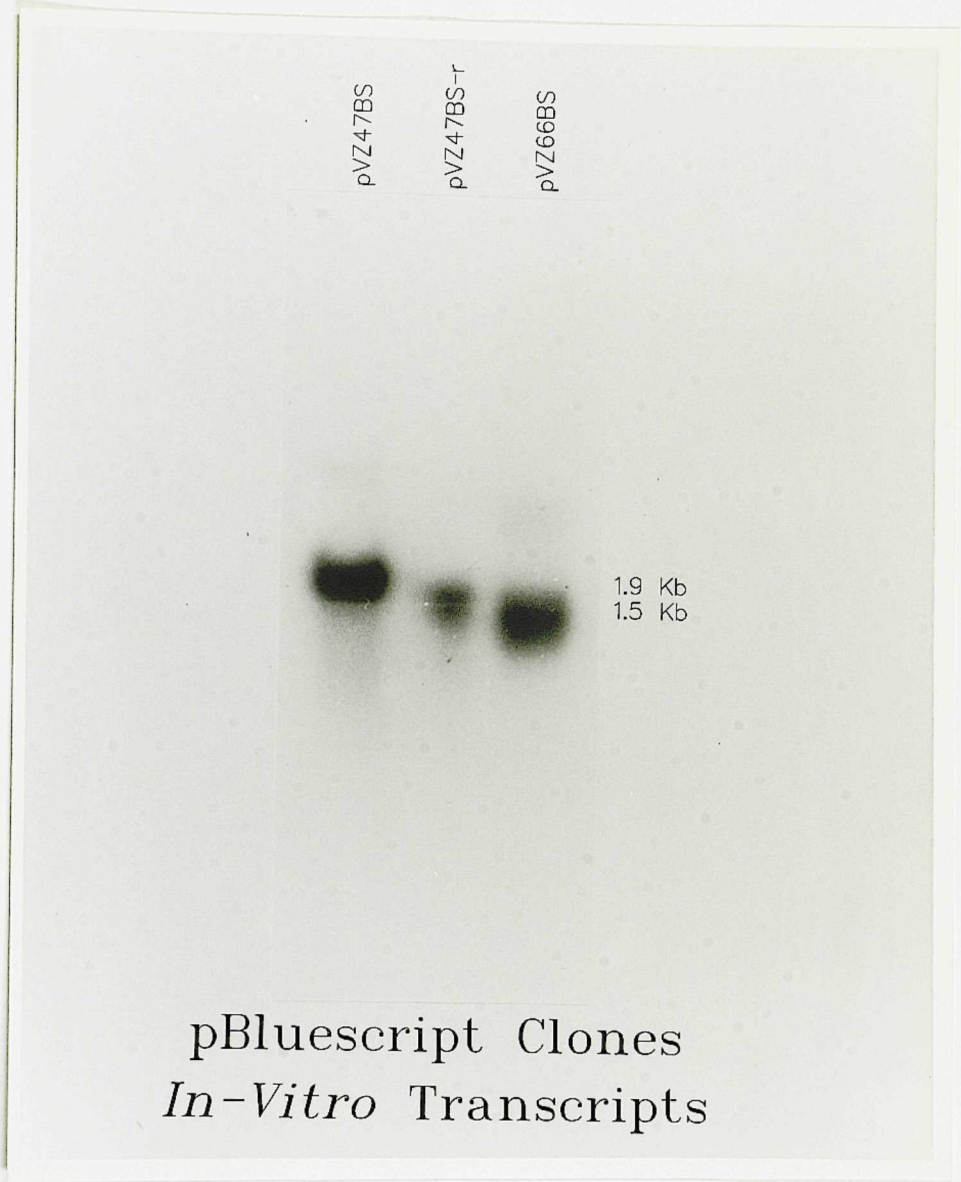


Figure 50. A photograph of a 1% (w/v) agarose gel, stained with ethidium bromide, of the DNA from the Bluescript clones of VZV genes 47 and 66. The expected sizes (Kb) of the fragments is indicated on the right.

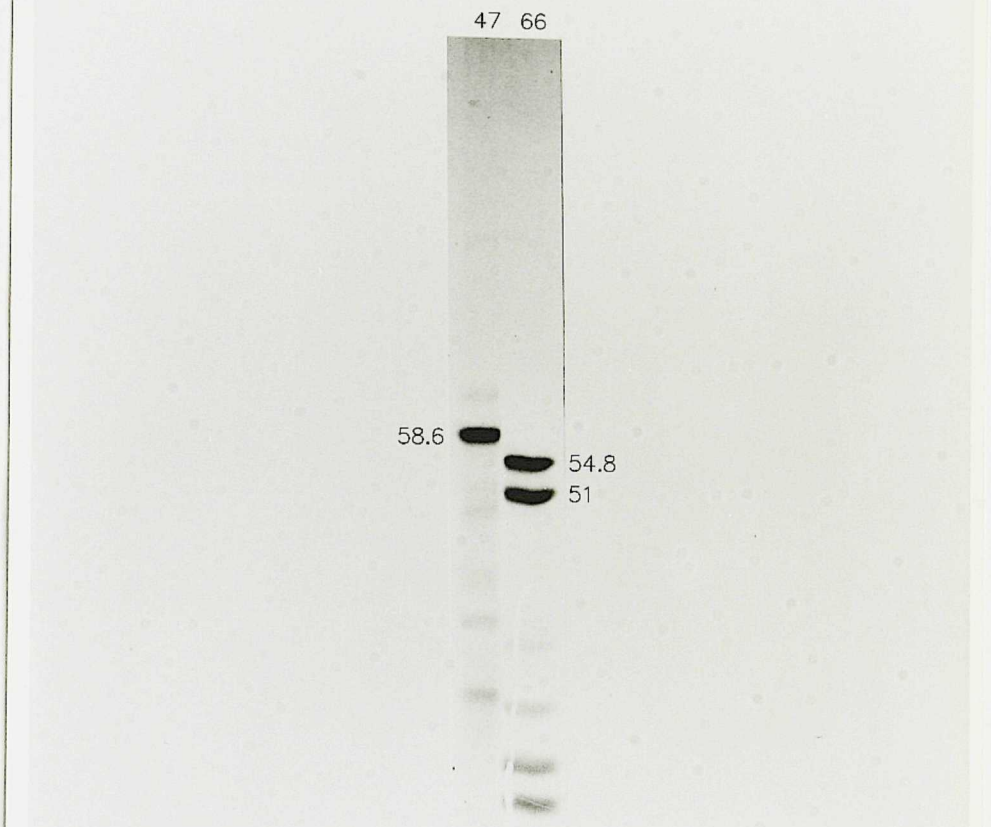
pBluescript Clones

Figure 51. An autoradiograph of the *in vitro* transcripts of the Bluescript clones of the VZV genes 47 and 66. The center lane is the result of transcription of the clone with gene 47 inserted in the reverse orientation. The transcripts were labelled by the addition of α^{32}P-UTP to the transcription reaction mix. The transcripts were run on an agarose gel with formaldehyde. Photograph kindly provided by Dr. Michael Pensiero.



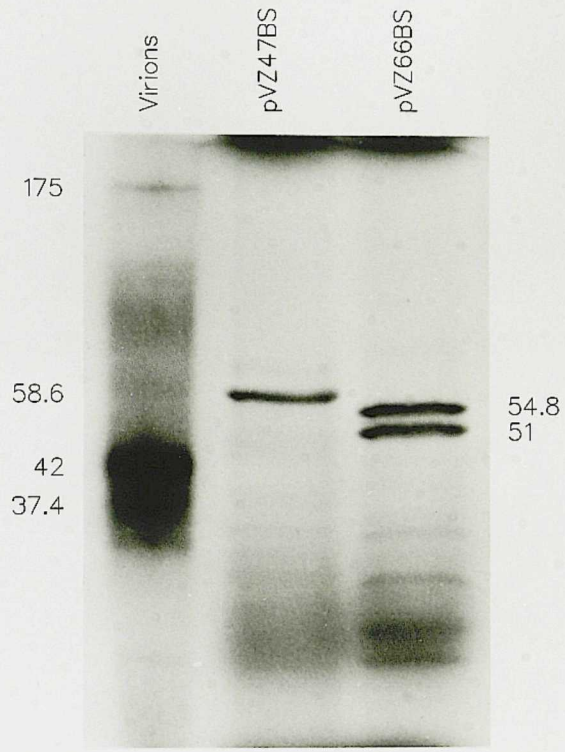
pBluescript Clones
In-Vitro Transcripts

Figure 52. An autoradiograph of an SDS-PAGE gel of the *in vitro* translation products of the Bluescript clones of VZV genes 47 and 66. The proteins were labelled by the addition of ^{35}S-Met to the rabbit reticulocyte translation mix. Labelled proteins kindly provided by Dr. Michael Pensiero. The molecular weights (kDa) are indicated alongside the bands.



pVZ47BS and pVZ66BS Translation Products

Figure 53. An autoradiograph of the ^{35}S labelled *in vitro* translation products of the Bluescript clones of VZV genes 47 and 66 run along side VZV virions labelled by the virion associated kinases. The molecular weights (kDa) are indicated alongside the bands.



VZV Kinase Reaction and
In-Vitro Translation Products

IV

DISCUSSION

The structure of Varicella-Zoster virus has not been extensively studied, due to the difficulty in purifying great enough quantities of the virus to work with. Much that is known about the structure of VZV has been inferred from studies of the other α herpesviruses, especially HSV-1. The goal of this dissertation was to carry out a series of studies on different aspects of the structure of VZV. The first of these was an examination of the core of the virion using ion etching, a unique technique which allows visualization of subviral components in an intact form distinct from the rest of the virion (Newcomb *et al.*, 1984). A second aspect was to identify whether several open reading frames in the VZV genome coded for structural proteins; the last aspect was to identify and characterize the protein kinase activity associated with purified VZV virions.

Virion Purification

To distinguish between the structural proteins of the virion and viral non-structural and cellular proteins, virions had to be purified to the greatest extent possible. Because of their highly cell-associated nature (Taylor-Robinson, 1959), purification and recovery of sufficient quantities of intact virions has traditionally been a difficult task. Following attempts at purification using several published techniques, we

settled upon the protocol used by Roberts (1984) as a basis for our purification scheme. This protocol uses a rate-zonal sucrose gradient to separate the virions from a cytoplasmic extract of infected cells and is dependent upon their sedimentation coefficient. This is followed by a glycerol/tartrate isopyknic gradient which separates the virions from other particles, based upon their density. Purification of VZV using the combination of both of these characteristics has proven highly effective in our hands.

On a purely qualitative basis, we knew from viewing samples of the purified virions on the electron microscope that they were quite clean (Figure 14). To quantitate the purity of the virions we added cells which had been labelled with ^{35}S-Met to infected cells and then purified the virions. Figure 16 summarizes the results from this experiment. Greater than 40% of the labelled proteins were removed just by separation of the nuclei from the cytoplasm of the cells. The sucrose gradient removed over half of the remaining labelled proteins, but they were still not clean enough since there remained many membranes and cellular debris (figure 13). The glycerol/tartrate density gradients were extremely effective in removing the majority of the remainder of the labelled proteins. There were less than 2% of the labelled proteins remaining after the first glycerol/tartrate gradient, and the appearance of negatively stained virions was quite good (figure 14). If a second gradient was done less than 1% of the labelled proteins remained, but the virions now appeared to be in very poor condition with many of them having disrupted membranes (figure 15). For our purposes we felt that the virions from the first glycerol/tartrate gradient were sufficiently purified and that attempts to

further purify them only lead to a decrease in the quality of the viral particles.

Using two different types of gradients separated the virions from contaminating material based on both their sedimentation coefficient and their density. This allowed us to achieve a greater than 60 fold purification of the virions which was comparable with the results achieved by Spear and Roizman (1972).

A comparison of the proteins of the purified virions with those of HFFs and HFFs infected with VZV on SDS-PAGE gels again showed that the purification procedure was effective in separating the virions from contaminating material (figure 17). The characteristic major tegument (175 kDa) and major capsid (155 kDa) proteins were readily apparent, and concentrated in the virions when compared with the infected cells. There were at least 38 proteins visible in the virions which was a greater number than had been reported previously (Shemer *et al.*, 1980). Some of these proteins could not be ruled out as cellular in origin due to the overlap in size of some of the cellular and viral proteins, but we felt that our protocol of using two types of gradients gave us cleaner virions than had been previously seen and that these were probably viral proteins.

The final criterion for purification was that we would be able to distinguish virion structural proteins from non-structural proteins. The method we used to test for this was comparison of virions with infected and uninfected cells on western blots. An example of western blots probed with antisera against both structural (ORF 10) and non-structural (ORF 29) is seen in figure 18. This showed that our virions were not contaminated with non-structural viral proteins.

The results of these experiments lead us to believe that we have purified intact VZV virions to the highest degree possible with the available techniques. We have demonstrated that we have separated the virions from both cellular and viral non-structural proteins while retaining the integrity of the viral particles.

Ion Etching

Herpesvirus-infected cell nuclei contain 3 types of nucleocapsid, based upon their density, designated light (L), intermediate (I), and heavy (H) (Perdue *et al.*, 1975). Studies on the maturation of the equine herpesvirus type 1 (EHV-1) showed that the light capsids were defective and accumulated in the nucleus, while the intermediates packaged the viral DNA and then matured into heavies, which were enveloped to become virions (Perdue *et al.*, 1976).

The core of the herpes virion has only been visualized by using thin sections of infected cells and purified virions (Furlong *et al.*, 1972; Nazerian, 1974; Friedmann *et al.*, 1975; Nii and Yasuda, 1975; Perdue *et al.*, 1976;Okada *et al.*, 1978). For HSV-1, it has been reported to be an electron-dense toroid, 50 nm high,with an 18 nm inner diameter and a 70 nm outer diameter, surrounding a cylindrical mass of lesser density (Furlong *et al.*, 1972). Similar structures have been observed, using the same techniques for several of the other herpesviruses (Nii and Yasuda, 1975).

Newcomb *et al.* (1984) developed a technique, called ion etching, which uses Ar^+ to etch away the exposed layers of viral particles. They have used this technique to look at the structure of the core of adenovirus (Newcomb *et al.*, 1984), to localize the structural proteins of

adenovirus (Newcomb and Brown, 1988) and HSV-1 (Newcomb and Brown, 1989), and to look at the packaging of the DNA in bacteriophage T4 (Black *et al.*, 1985) and bacteriophage λ (Brown and Newcomb, 1986). This technique is unique in that it etches away exposed surfaces while leaving underlying structures intact.

We have used this technique to look at the core of the VZV virion. The ideal experiment would be to compare light, intermediate, and heavy VZV nucleocapsids, but we were never able to purify the nucleocapsids in quantities great enough to do this experiment. There seems to be some innate difference between HSV and VZV nucleocapsids since HSV nucleocapsids could be purified readily while VZV nucleocapsids could not. Therefore whole VZ virions were used for the experiment.

Using whole virions for the experiment made interpreting the results more difficult due to the variability of the sizes and content of the virions. The sizes varied apparently due to the amount of tegument present and the state of the viral membrane. Virions with intact membranes were more or less spherical, while those with disrupted membranes spread out much like a fried egg. This caused variation in the way the tegument was presented to the Ar ions during etching, which caused variation in the time it took to etch down to the core of each virion. If we had started with nucleocapsids there would have been much less variation in the time it took to expose the cores.

A statistical analysis of the measurements of the particle sizes at the different time points clears up the confusion of the variability caused by using whole virions. The analysis of the distribution of sizes at each time point showed that there was a steady shift in the size

distribution toward the size of the core as the time of etching increased (figure 20). The χ^2 analysis of this data also showed that there was a highly significant correlation between the time etched and the distribution of the sizes of the particles.

The analysis of variance and a plot of the means of the diameters at each time point revealed that there were 3 stages of etching during the process (figure 19). The first time period gave a significant drop in size probably due to the loss of the viral membrane and its glycoproteins. The next stage was the etching away of the tegument of the virions. It took 20 secs. for another significant drop in size, which was probably due to a resistance in the tegument to being etched away. This resistance was probably due to the fixation of the tegument proteins indicating they probably form a compact structure in the intact virion. This was followed by the removal of the layers of the nucleocapsid to reveal the core. The capsid also appeared to be resistant to etching which probably indicated a tightly organized structure. Even though the drop between 30 and 40 sec. was statistically significant it showed a decrease in the rate of etching which may support the presence of a second icosahedral shell within the nucleocapsid (Schrag *et al.*, 1989).

Cores appeared as early as 30 sec. because there were a few free nucleocapsids among the virions used for etching. At the 40 sec. time point many more could be seen, many with bits of capsid material still on them. At the 50 sec. time point there were many cores visible. They were seen in all orientations on the grid due to the random orientation of the capsids they were in. This random orientation made it difficult to measure their dimensions. Most appeared to be 30-40 nm in height which

was not as thick as those seen by Furlong *et al.* (1972). Their other dimensions were much the same though. They had a diameter of 50-70 nm and an inner diameter of about 15-20 nm. The interesting thing visible by this technique was that several of them appeared to be open ended instead of a complete toroid.

It has been proposed in the past that the DNA of the virion is wound around the central mass, seen in thin sections, to form the toroid (Perdue *et al.*, 1976). If indeed the toroid is open ended a different model for packaging the DNA must be proposed. The mechanics involved in winding the DNA around a central mass would be much more complex than if the DNA is first condensed and then wrapped around the central mass.

The appearance of the particles we have observed by ion etching the virions, and the statistical analysis of the sizes of the particles produced by the etching leads us to believe that we have isolated the core of the VZV virion. But, without being able to show that these structures are composed of DNA we can not say this with absolute certainty. Therefore, further work needs to be done to show the presence of DNA following etching. First, the etching needs to be repeated with virions which have been grown in medium with ^{3}H-thymidine so that their DNA is labelled. Then the radioactivity could be measured at each time point so that it could be shown that DNA is present at the point where the cores are seen, and that it decreases as the cores are etched away. Another technique to show the presence of DNA at the final time point is to etch virions on coverslips, recover the DNA from them, and use it to make random primer labelled probes for Southern blots of whole VZV DNA.

So far all of the herpesvirus nucleocapsids looked at in thin

sections have contained toroids (Nii and Yasuda 1975). There have been some variations observed between the different viruses, but basically they all have the same structure. From this it would be expected that the core of HSV is relatively the same as that for VZV. Therefore, since full and empty HSV nucleocapsids can be isolated they should be etched and compared to show that the structures we are calling cores are not present in the empties.

Molecular Cloning Into Vaccinia Virus

An important part of the study of the structure of VZV is going to be the identification of all of the structural proteins of the virion. As noted earlier, when purified virions are looked at on an SDS-PAGE gel there are many proteins which can not be specifically identified as viral. In order to unequivocally identify them they are all going to have to be mapped to the viral genome.

The extreme difficulty of making VZV mutants makes classical genetic mapping too cumbersome to do. A technique for mapping, which has been successfully used in our lab, is to clone VZV genes into vaccinia virus under the control of a vaccinia promoter (Chakrabarti *et al.*, 1985). The vaccinia recombinants, which express the VZV gene, can be used to immunize rabbits to get antibody specific for the VZV gene product (Kinchington *et al.*, 1990).

As described in the results section, several VZV genes were picked as putative structural genes. One gene, gene 40 was known to be the major capsid protein, but the rest were picked based upon their homology with HSV genes. Recently, gene 47 has been identified as a putative protein kinase based on the presence of the eukaryotic protein

kinase domain as part of its sequence.

Genes 11, 33, 34, 40, 47, and 66 were successfully cloned into pSC11, the vector used to insert the genes into vaccinia virus. Of these, genes 33, 34, 40, and 47 were inserted into the vaccinia genome. After confirming the presence of these genes in the recombinants, they were used to immunize rabbits. Rabbit sera were screened for the presence of antibodies using western blots of uninfected cells, cells infected with VZV, cells infected with the vaccinia recombinants, and purified VZV. After several injections, the rabbits developed antibodies against vaccinia proteins but not any VZV proteins. There were also no signs of infection at the site of injection. It turned out that there was a misunderstanding about the administration of the virus, and it had been mixed with Freund's adjuvant prior to injection. We feel this must have killed the virus so the rabbits were never exposed to any of the VZV proteins.

A new series of rabbits was started, and this time there was swelling, redness, and lesion formation at the site of the injections. So far the rabbits have produced a high titer of antibody against the vaccinia proteins, and some antibodies appear to have been raised against VZV proteins (figure 39). The VZV proteins seen are not in the size range expected for the genes we have cloned. Also, 2 of the rabbits appear to be developing a very similar set of antibodies even though they have been injected with different antigens (figure 39). Since the constructs were checked again by southern blot for the presence of the VZV genes prior to the injection of the second set of rabbits, it appears there may have been a mix up of the samples given the rabbits.

Following the previous success this laboratory has had using this

technique, we are quite puzzled by the fact that these clones have not worked. Care was taken to insure that these genes were inserted in the proper orientation and that there were no extraneous translation start sites between the promoter and the gene. There is an unlikely possibility that the pSC11 used for the cloning has a mutated p7.5 promoter, and therefore the genes are not being transcribed. We did not do northern blot analysis of the recombinants because of our confidence from previous successes using this technique and time limitations.

Several things remain to be done. First, the recombinants need to be checked to make sure they are not contaminated with revertants. This is unlikely because recent southern blots show the presence of the cloned genes in the recombinants. Next, northern analysis needs to be done to insure the genes are being transcribed. Following this, new rabbits need to be immunized under our direct supervision, and their sera screened not only with western blots, but also by immunofluorescence of VZV infected cells and immunoadsorption of VZV proteins from infected cells.

A lack of success with making antibody with these recombinants is in no way an indictment against this technique. It has been highly successful in the past and is still an excellent tool for looking at the proteins produced by VZV and mapping them to their genome. Some unforeseen factor has interfered with the expression of these genes, which we are confidant will be found and corrected.

Once antibody is obtained using these clones many experiments concerning the structure of the virion will be done. The first thing shown will be whether these proteins are structural. The antibodies will

then be used to study the proteins within infected cells by fluorescence. Also the antibodies could be used to probe western blots of virions at different stages of ion etching to localize the protein within the virion. All of these things will aid in our understanding of the structure of VZV.

VZV Associated Protein Kinase Activity

As more is learned about eukaryotic protein kinases the importance they play in the regulation of protein activity is ever increasing. A nice analogy to electronics is that they are "the transistors of the cell" (Hunter, 1987). They all posses an approximately 240 amino acid catalytic domain which has 11 highly conserved motifs within it (Hanks *et al.*, 1988). The presence of this domain has allowed several putative protein kinases to be identified within the herpesvirus family (Smith and Smith, 1989).

Protein kinase activity associated with viruses was first reported by Strand and August in 1971. They noted that it appeared to be associated with enveloped viruses but not those without an envelope. Over the years many people have investigated the kinase activities associated with many different viruses, but so far, the only true viral kinases have been found in the α herpesvirus family (Leader and Katan, 1988) and vaccinia virus (Howard and Smith, 1989). The kinases associated with retroviruses (Collett and Erikson, 1978), being acquired accidentally from their host cells, appear to only be transient hitchhikers and can not be truly classified as viral (Duesberg, 1983).

Herpes simplex virus was shown to have kinase activity associated with its enveloped virions but not the nucleocapsids (Rubenstein *et al.*, 1972). It was also shown, using equine herpesvirus (EHV), that the mem-

brane of the virion had to be disrupted using NP40 before labelling of viral proteins by the addition of γ^{32}P-ATP could take place (Randall *et al.*, 1972).

Soluble extracts were made from the purified virions of pseudorabies virus (PRV) and HSV and were shown to contain 2 different kinase activities by substrate specificity. One of the kinases had activity characteristic of cellular casein kinase II, while the other had characteristics unique from known cellular kinases (Stevely *et al.*, 1985).

HSV infected cells were shown to posses a protein kinase not found in uninfected cells (Purves *et al.*, 1986). This kinase was partially purified from the cells and shown to be the product of HSV gene U_S3 by antibody made against the carboxy terminal 8 amino acids of this protein (Frame *et al.*, 1987). It was also shown by Purves *et al.* in 1987 that an HSV-1 mutant with a deletion in gene U_S3 grew normally in cell culture and the infected cells no longer possessed the kinase. Up until recently, when it was shown that HSV-1 gene U_L13 possessed a protein kinase domain (Smith and Smith, 1989), it has been assumed that the protein kinase associated with purified virions has been the U_S3 gene product (McGeoch and Davison, 1986).

VZV genes 47 and 66 are homologous to HSV-1 genes U_L13 and U_S3 respectively. Using the GCG BestFit program to compare the sequences of these proteins shows that gene 47 and U_L13 have greater than 55% similarity and genes 66 and U_S3 have almost 58% similarity. The interesting thing is that the program perfectly aligns their kinase domains while a comparison between 47 and 66 or U_L13 and U_S3 do not.

We have been studying the kinase activity associated with the

purified virions of VZV. When they are incubated with γ^{32}P-ATP in the presence of NP40 there are 3 major proteins labelled along with many minor proteins (figure 40). One of these proteins is the 175 kDa major tegument protein IE62 which is the HSV-1 ICP4 equivalent. This protein has been shown to play a major role in the trans-activation of viral genes during infection. It is interesting to note that HSV-1 does not possess a similar structural gene, and VZV does not possess an active αTIF which in HSV-1 is a structural protein responsible for induction of its α genes. The other 2 major phosphorylated proteins (42 and 37.4 kDa) have not been identified, but are similar in size to proteins identified by Roberts in 1984 as being DNA binding proteins. This leads us to believe that a virion kinase may play a role in the regulation of viral transcription.

A soluble extract was made from purified VZV which contained kinase activity. This extract could be added to heat-inactivated virions reconstituting their kinase activity. When the extract was fractionated using Amicon filters 2 different kinase activities were revealed (figure 41). The fraction retained by the C-100 filter no longer phosphorylated the 37.4 kDa protein while the C-30 fraction did. It is assumed that the C-30 filter retains both kinases while the C-100 fraction allows one to pass through. The one kinase may form aggregates with other proteins or since kinases are phosphoproteins themselves it may be possible that there is only one kinase present in 2 forms, the phosphorylated form having a greater charge may interact with the C-100 membrane while the unphosphorylated form passes through.

Whole virions, along with infected and uninfected cells, were run on SDS-PAGE gels and blotted to immobilon membranes. Following renatur-

ation the membranes were put into buffer with γ^{32}P-ATP to allow any kinases present to autophosphorylate. The results showed that there are 2 protein kinases present in the virions (figure 46). One of these is a cellular kinase with a molecular weight of around 82 kDa while the other is a viral kinase of around 55 kDa. This is approximately the size of the gene 66 *in vitro* translation product. It is interesting to note that several of the cellular kinases appear to have been shut down (50 kDa), or greatly reduced (54 kDa) by the viral infection, but not all (figure 46).

We have cloned both gene 47 and gene 66 into the pBluescript plasmid which allowed us to transcribe and translate the genes *in vitro*. When they were translated gene 47 gave a 58.6 kDa product and gene 66 gave 54.8 and 51 kDa products. The presence of two products from the *in vitro* translation of gene 66 was probably due to the presence of a second translation start site 91 nucleotides downstream from the first start site. When these products were labelled and run alongside virions from the kinase reaction no apparent bands corresponding to them appeared in the virions, but they were in a region where there are many minor virion phosphoproteins. Based upon the size of the virion kinase seen in the autophosphorylation experiment the upper gene 66 band is probably the virion kinase.

In an attempt to show that the translation products were kinases, they were added to heat inactivated virions. The products had been made using the rabbit reticulocyte system, and were added to the virions along with the lysate. It was then that we found that the rabbit reticulocyte lysate is inhibitory for the kinases found associated with the virions. Rabbit reticulocyte lysate contains 2 kinases, one of which is activated

by dsRNA and the other which is inhibited by heme (Farrell *et al.*, 1977).

Several experiments were done to try to get rid of the inhibitory factor in the lysate. Spin columns and dialysis were not effective, but dilution of the lysate did get rid of inhibition. But since the products of the lysate reaction are at such low levels, if we were to see their activity, dilution was not the best method to use. We therefore tried the Amicon filters and found that the inhibitor could be removed with the C-30 filter. But this also allowed the lysate kinase(s) to become active, and it phosphorylated all 3 of the major virion phosphoproteins. We next tried putting the lysate through a C-100 filter and then a C-30 filter, using the retentate from the C-30 filter. This got rid of the inhibitor and also the lysate kinase. From these experiments it appears that the inhibitor of the virion kinases may be the same as for the lysate kinase, which is heme (Farrell *et al.*, 1977). In order to prove this we are going to have to add heme to the kinase reaction.

There are several experiments which need to be done before this project is finished. We have decided to search the sequences of genes 47 and 66 for antigenic regions and make synthetic peptides of them in an attempt to make antibodies. (We have injected rabbits with the *in vitro* translation products from both genes with no success so far.) This is the only way we will be able to positively identify the viral protein kinase(s) present in the virions.

While antibody is being made there are experiments which can be done to further characterize the translation products of the kinase genes. The first is an attempt to autophosphorylate them as was done with the virions. This may be difficult due to the small amount present in the

translation reactions, but since there is probably very little present in the virions we feel it will work. Next, the products may be able to be separated from the lysate kinases using the Amicon filters. If translation products do not stick to the C-100 membrane we will be able to add them to heat inactivated virions to see if they are active kinases, and the pattern of phosphorylation they exhibit will give us help in determining which is present in the virion.

This will complete this project, but it is just the beginning for much more work. The proteins of the virion which are phosphorylated need to be investigated as to their functions. It is hard to imagine something as highly efficient as a virus packaging an enzyme as part of its virion for no reason. This must play some important role in the replicative cycle of the virion or it would not be present. The key to its role is the proteins phosphorylated by it.

APPENDIX

Diagrams of Plasmid Constructs

Figure 54. The cloning of p9VZ11. The 2,769 bp Bsp 1286/Mlu I fragment from the VZV KpnC clone was inserted into the Sma I site of pUC9 to give p9VZ11.

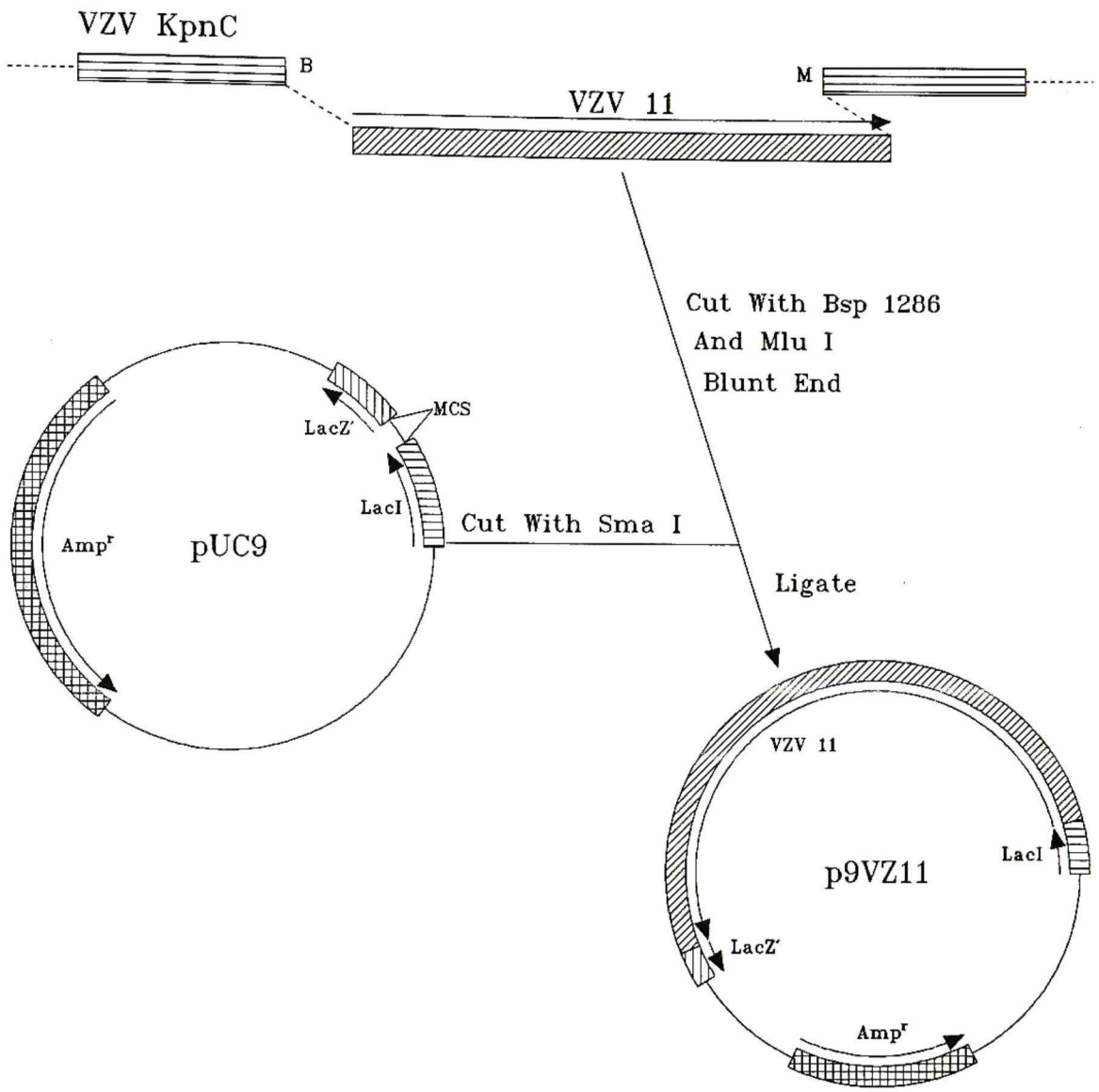

VZV KpnC
B
M
VZV 11
Cut With Bsp 1286
And Mlu I
Blunt End
MCS
LacZ'
LacI
Amp^r
pUC9
Cut With Sma I
Ligate
VZV 11
LacI
p9VZ11
LacZ'
Amp^r

Figure 55. The cloning of pVZ11SC. The 2,769 bp Bsp 1286/Mlu I fragment from the VZV KpnC clone was inserted into the Sma I site of pSC11 to give pVZ11SC.

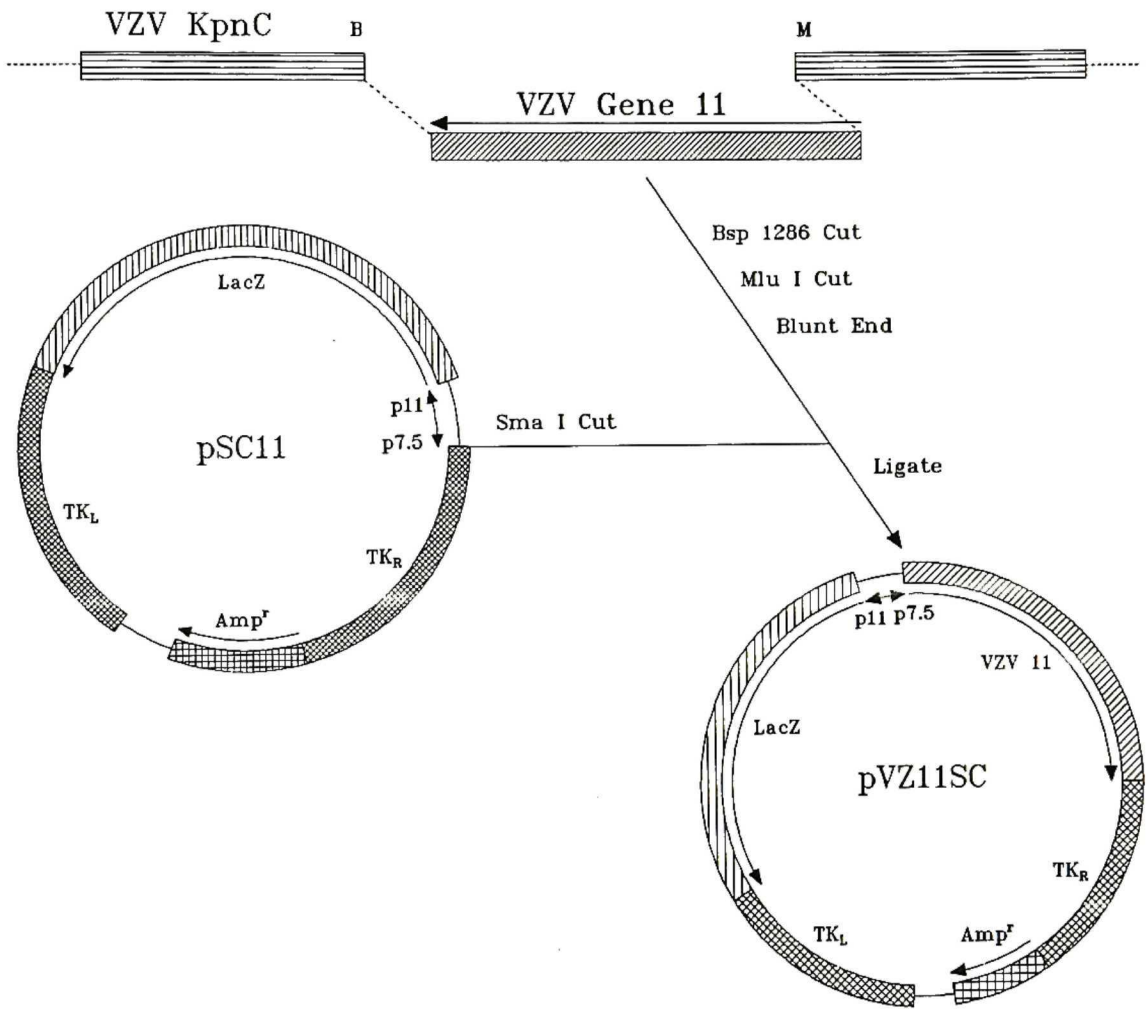

VZV KpnC
B
M
VZV Gene 11
Bsp 1286 Cut
Mlu I Cut
Blunt End
LacZ
p11
p7.5
pSC11
Sma I Cut
Ligate
TK$_L$
TK$_R$
Amp^r
p11 p7.5
VZV 11
LacZ
pVZ11SC
TK$_R$
TK$_L$
Amp^r

Figure 56. The cloning of p9VZ33. The 2.058 bp Ban I/Dde I fragment of the VZV KpnD clone was inserted into the Sma I site of pUC9 to give p9VZ33.

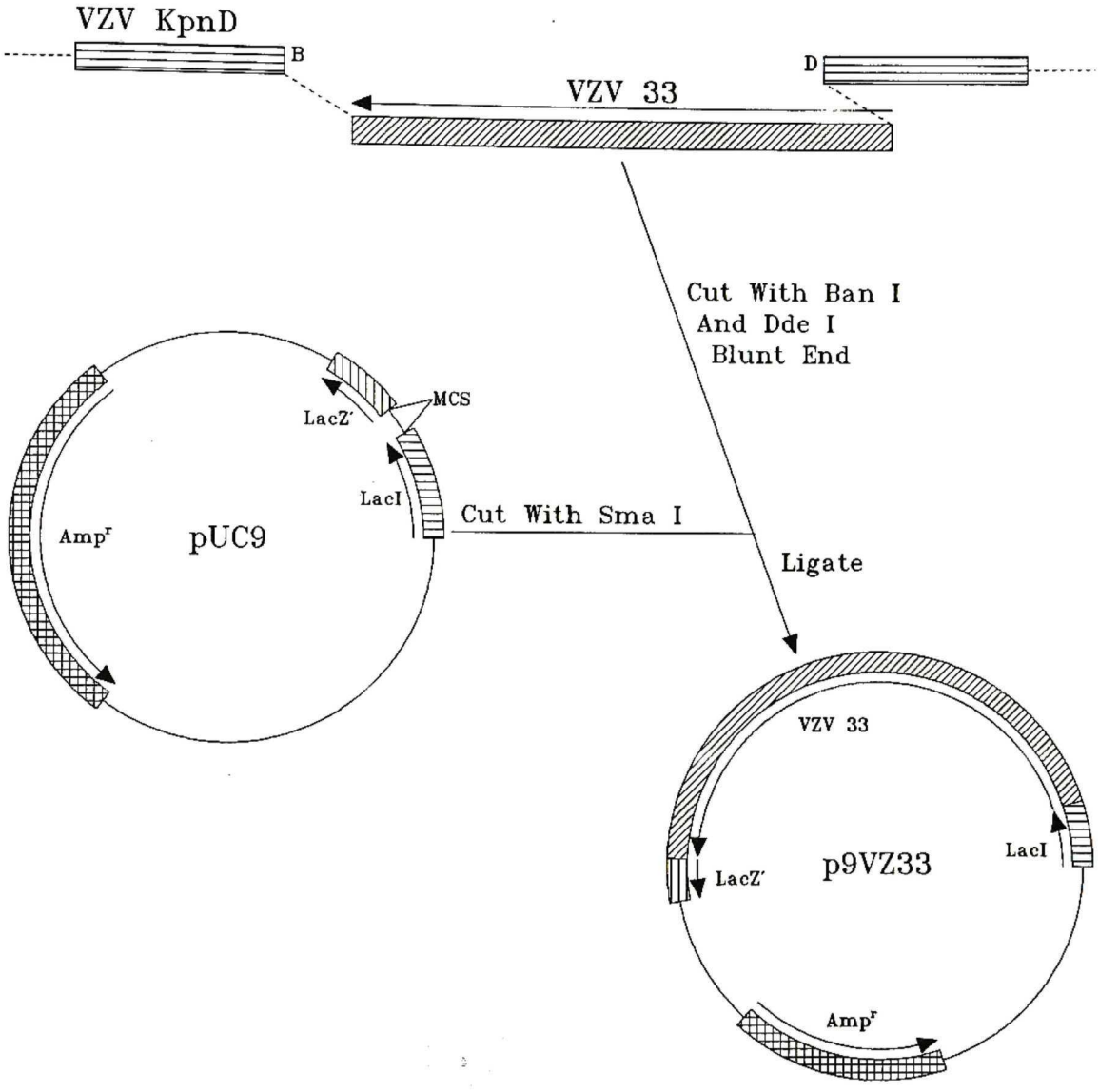

VZV KpnD
B
D
VZV 33
Cut With Ban I
And Dde I
Blunt End
MCS
LacZ'
LacI
Amp^r
pUC9
Cut With Sma I
Ligate
VZV 33
LacZ'
p9VZ33
LacI
Amp^r

Figure 57. The cloning of pVZ33SC. The 2,058 bp Hind III/Eco RI fragment from p9VZ33 was inserted into the Sma I site of pSC11 to give pVZ33SC.

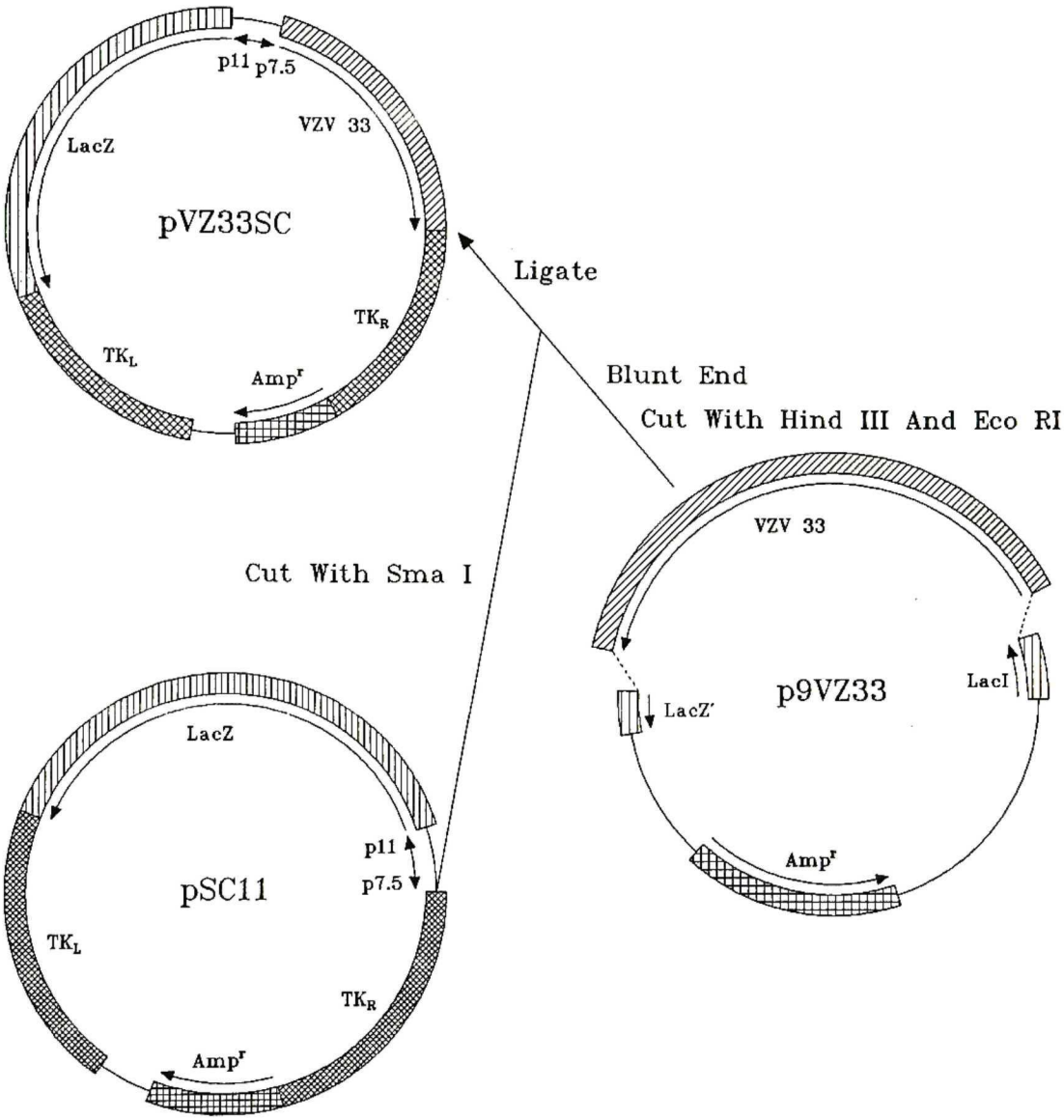

p11
p7.5
VZV 33
LacZ
pVZ33SC
TK$_R$
TK$_L$
Amp^r
Ligate
Blunt End
Cut With Hind III And Eco RI
Cut With Sma I
VZV 33
LacZ'
p9VZ33
LacI
Amp^r
LacZ
p11
p7.5
pSC11
TK$_L$
TK$_R$
Amp^r

Figure 58. The cloning of p9VZ34. The 2,052 bp Cla I/Stu I fragment from the VZV BamH clone was inserted into the Sma I site of pUC9 to give p9VZ34.

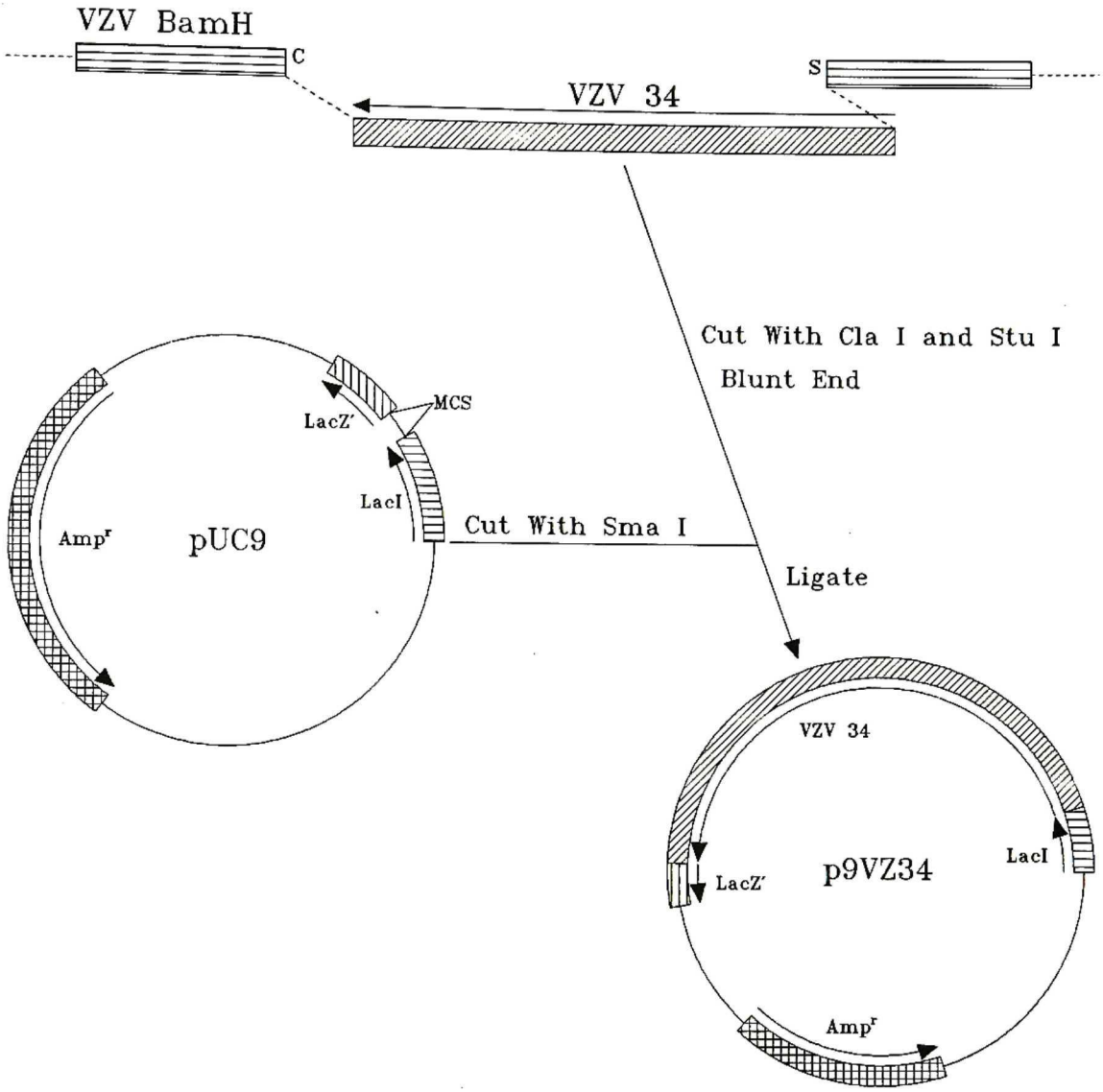

VZV BamH
C
S
VZV 34
Cut With Cla I and Stu I
Blunt End
LacZ'
MCS
LacI
Amp^r
pUC9
Cut With Sma I
Ligate
VZV 34
LacZ'
p9VZ34
LacI
Amp^r

Figure 59. The cloning of pVZ34SC. The 2,052 bp Cla I/Stu I fragment from the VZV BamH clone was inserted into the Sma I site of pSC11 to give pVZ34SC.

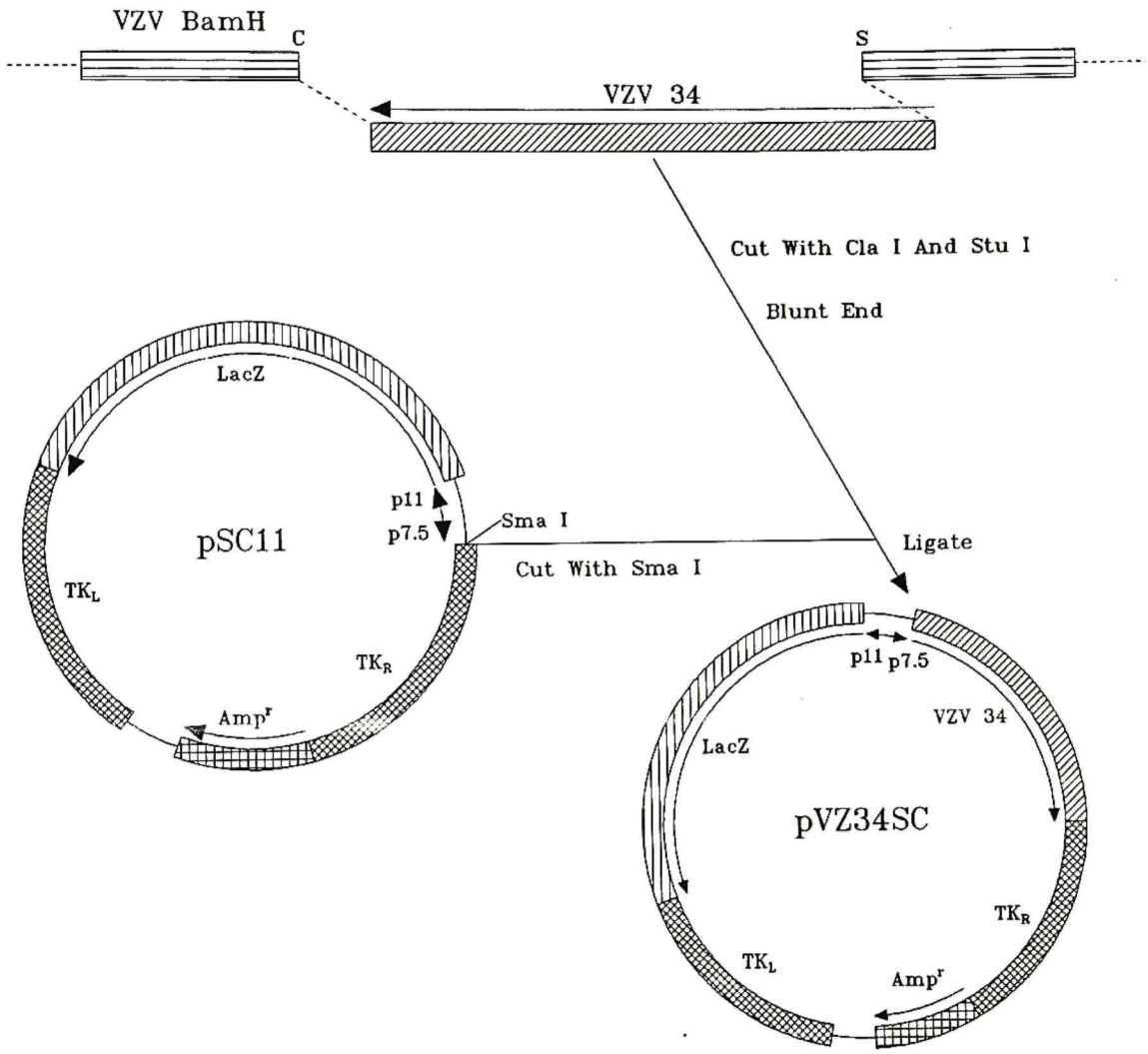

VZV BamH
C
S
VZV 34
Cut With Cla I And Stu I
Blunt End
LacZ
p11
p7.5
pSC11
Sma I
Cut With Sma I
Ligate
TK_L
TK_R
Amp^r
p11 p7.5
VZV 34
LacZ
pVZ34SC
TK_R
TK_L
Amp^r

Figure 60. The cloning of pVZ40BSC. The 4,795 bp Bst XI fragment of the VZV EcoD clone was inserted into the Sma I site of pSC11 to give pVZ40BSC.

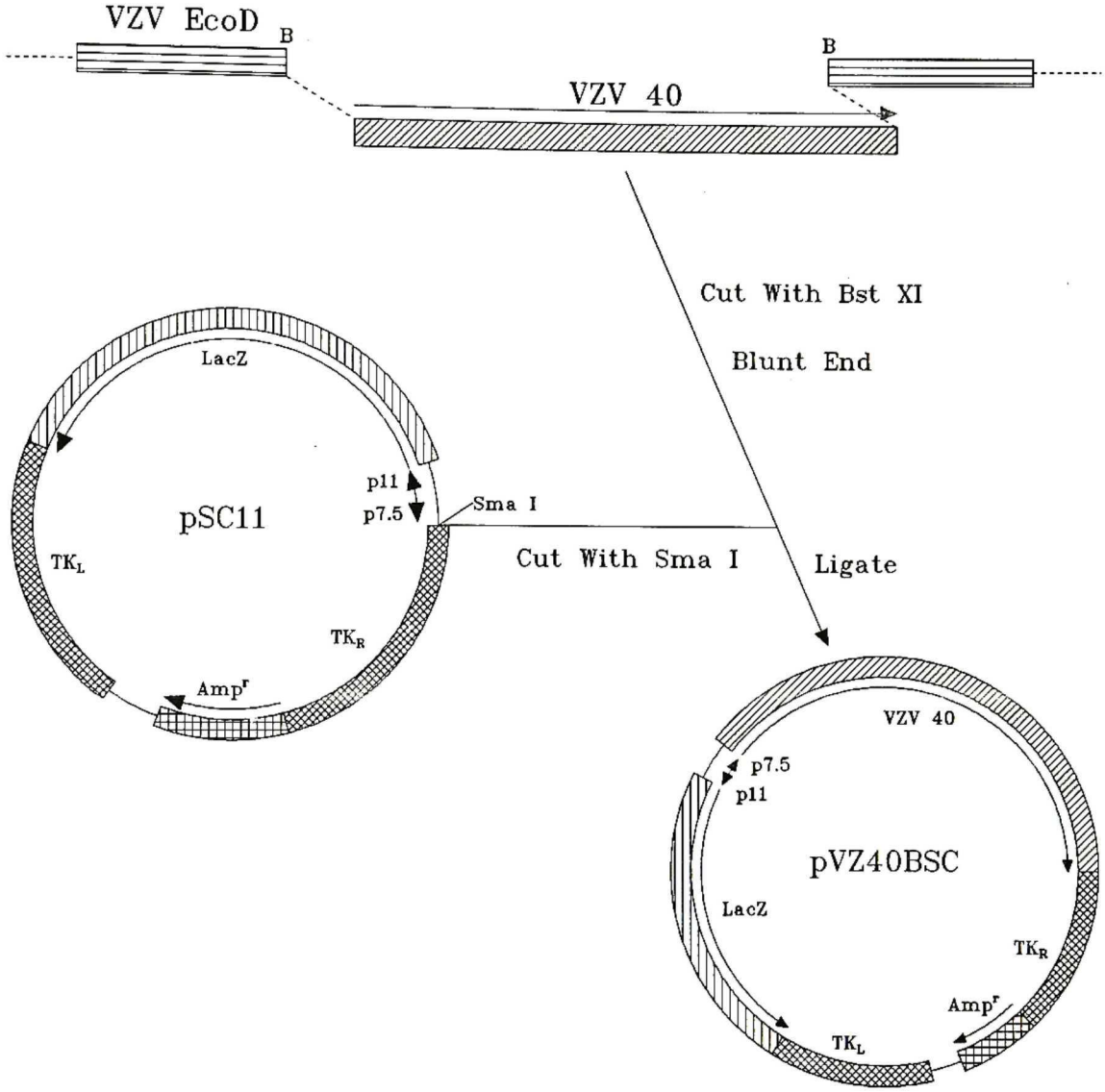

VZV EcoD
B
B
VZV 40
Cut With Bst XI
Blunt End
LacZ
p11
p7.5
pSC11
Sma I
Cut With Sma I
Ligate
TK$_L$
TK$_R$
Amp^r
VZV 40
p7.5
p11
pVZ40BSC
LacZ
TK$_R$
Amp^r
TK$_L$

Figure 61. The cloning of pHX4. The 275 bp Hpa II/Xba I fragment of the VZV EcoD clone was inserted into pBS, which had been cut with Acc I and Xba I, to give pHX4. Hpa II can insert into an Acc I site with the loss of the site for both enzymes.

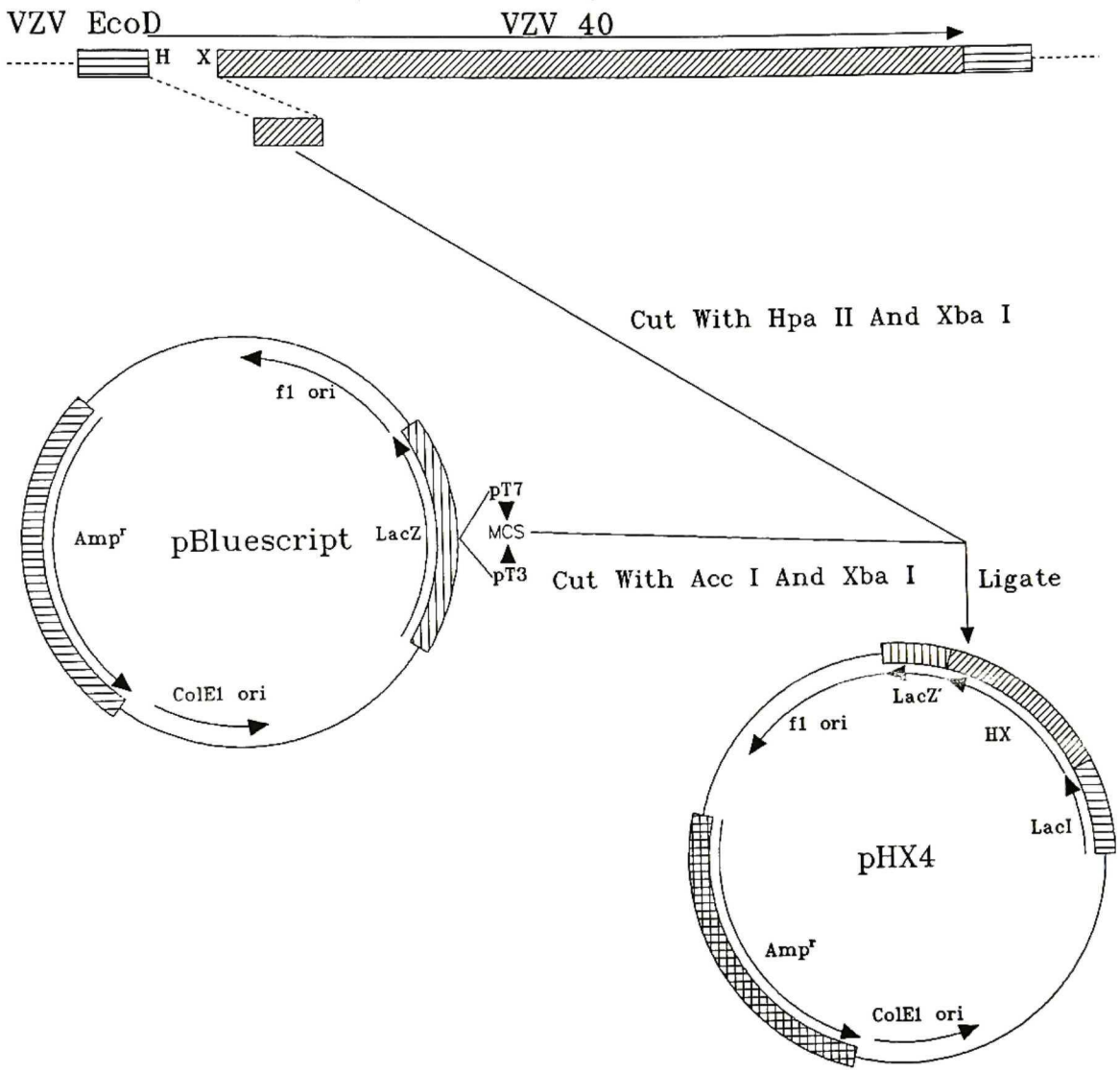

VZV EcoD
VZV 40
H
X
Cut With Hpa II And Xba I
f1 ori
Amp^r
pBluescript
LacZ
pT7
MCS
pT3
ColE1 ori
Cut With Acc I And Xba I
Ligate
LacZ'
f1 ori
HX
LacI
pHX4
Amp^r
ColE1 ori

Figure 62. The cloning of pHX4'. The 4,906 bp Xba I fragment from the VZV EcoD clone was inserted into pHX4, which had been cut with Xba I, to give pHX4'.

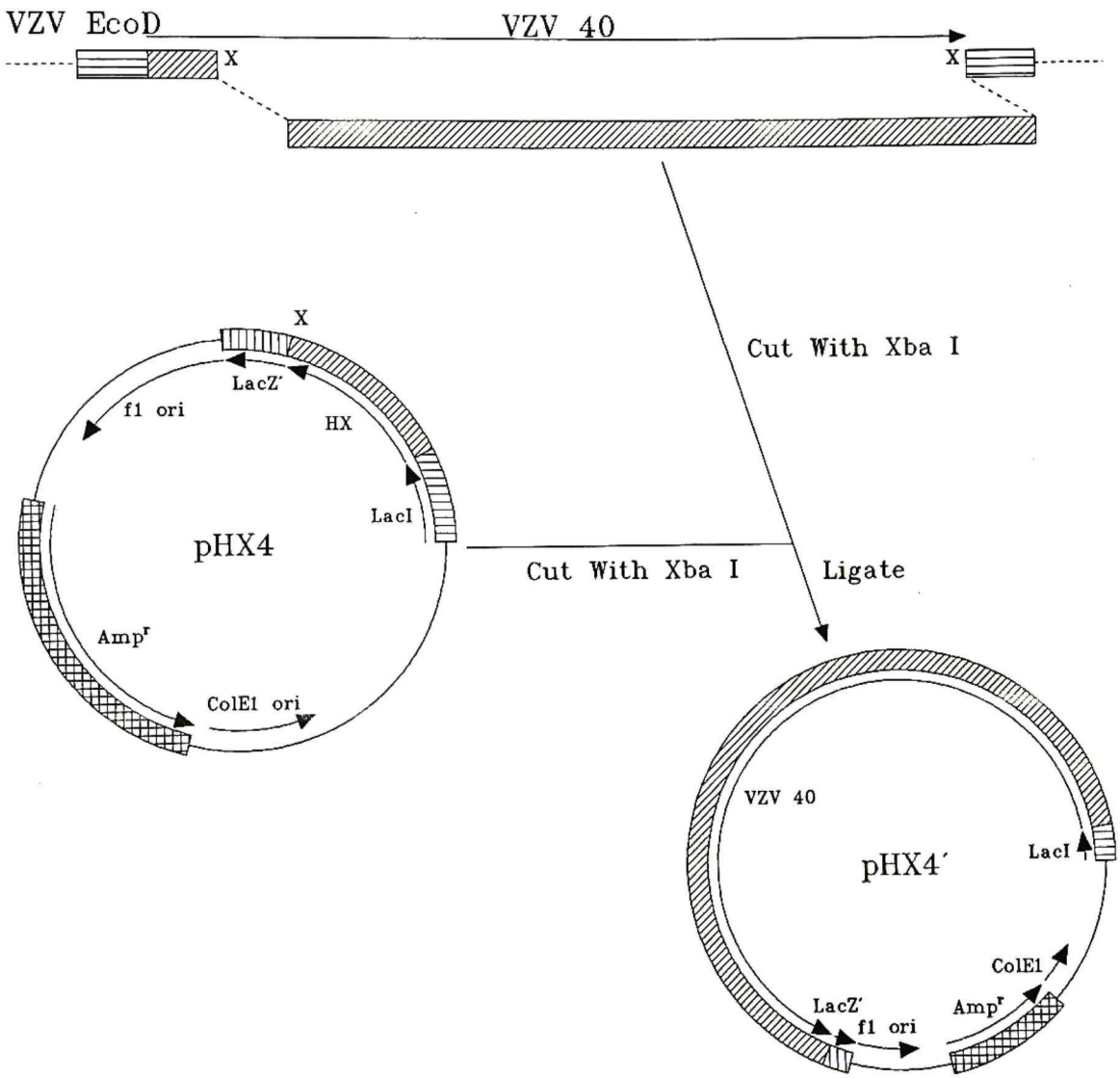

VZV EcoD
VZV 40
X
X
Cut With Xba I
X
LacZ'
f1 ori
HX
LacI
pHX4
Cut With Xba I
Ligate
Amp^r
ColE1 ori
VZV 40
pHX4'
LacI
ColE1
LacZ'
f1 ori
Amp^r

Figure 63. The cloning of pVZ40HXSC. The 5,131 bp Hind III/Eco RI fragment from pHX4' was inserted into the Sma I site of pSC11 to give pVZ40HXSC.

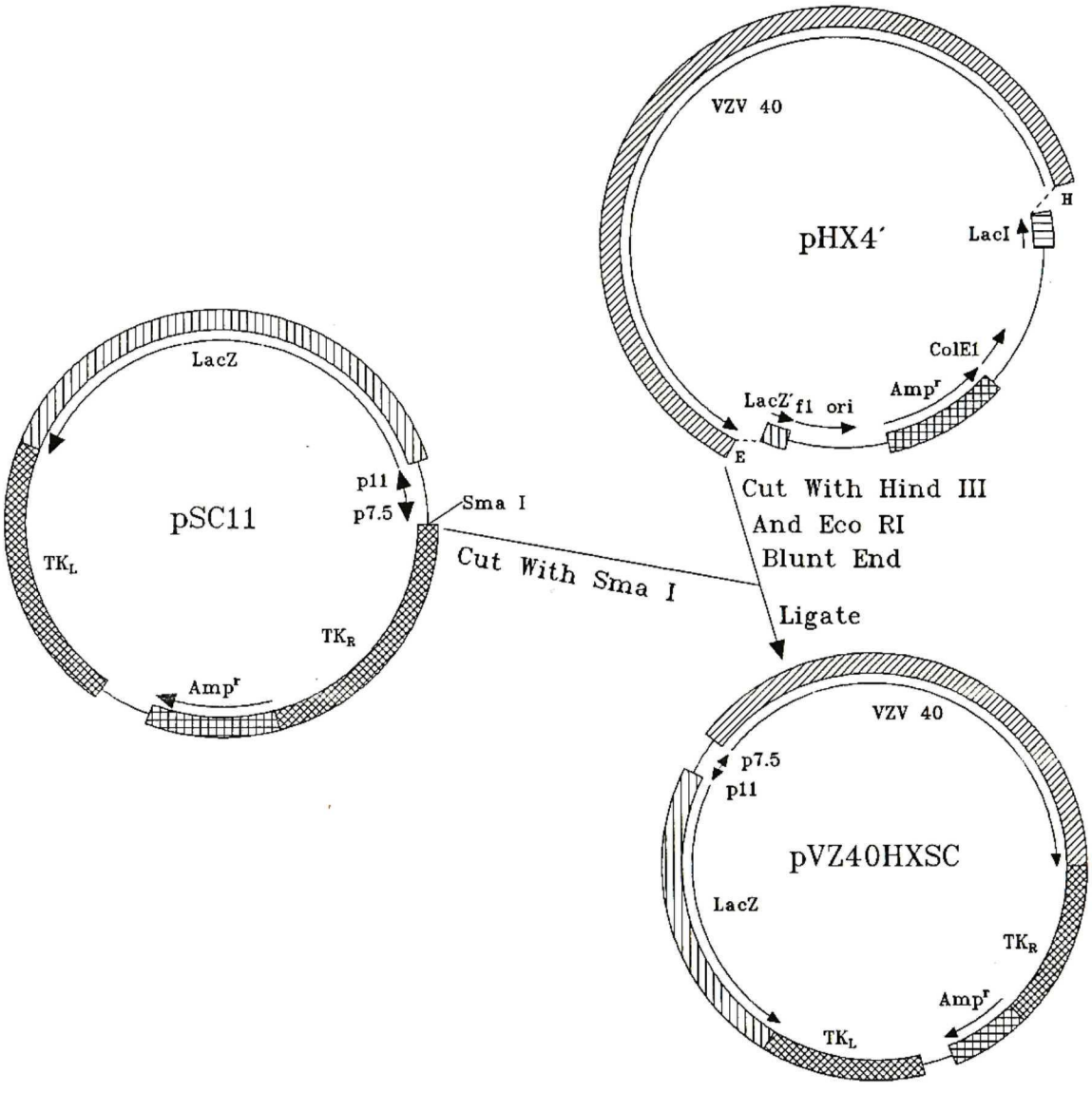

VZV 40
pHX4´
H
LacI
ColE1
Amp^r
LacZ´ f1 ori
E
Cut With Hind III
And Eco RI
Blunt End
Ligate
LacZ
pSC11
p11
p7.5
Sma I
Cut With Sma I
TK_L
TK_R
Amp^r
VZV 40
p7.5
p11
pVZ40HXSC
LacZ
TK_R
Amp^r
TK_L

Figure 64. The cloning of p9VZ40P. The 2,163 bp Pst I fragment of pHX4' was cloned into the Pst I site of pUC9 to give p9VZ40P.

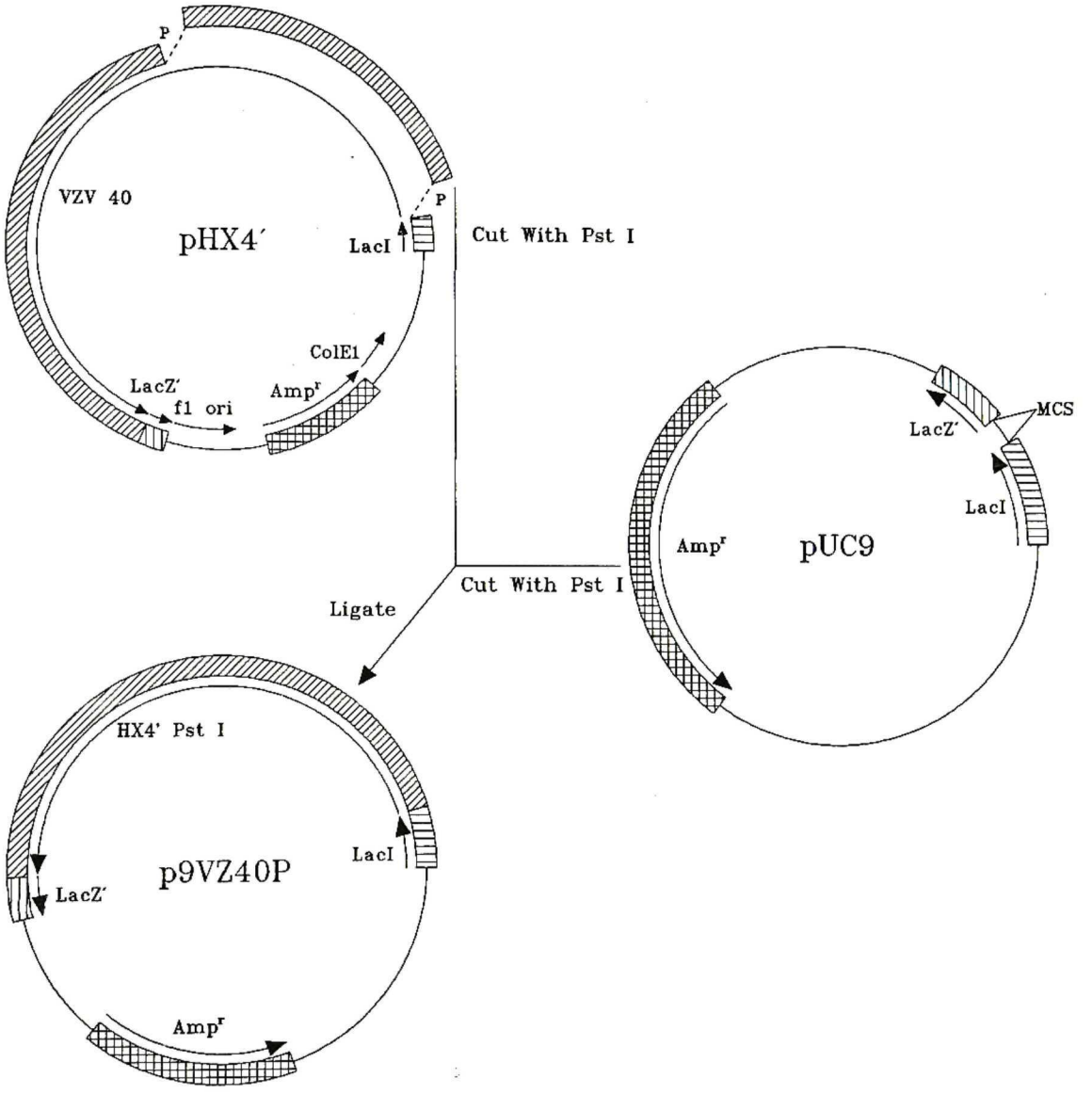

P
VZV 40
pHX4′
P
LacI
Cut With Pst I
ColE1
LacZ′
f1 ori
Amp^r
LacZ′
MCS
LacI
Amp^r
pUC9
Cut With Pst I
Ligate
HX4′ Pst I
LacI
p9VZ40P
LacZ′
Amp^r

Figure 65. The cloning of p9VZ40. The 3,751 bp Nae I/Eco RI fragment of pHX4' was inserted into p9VZ40P cut with Nae I and Eco RI to give p9VZ40.

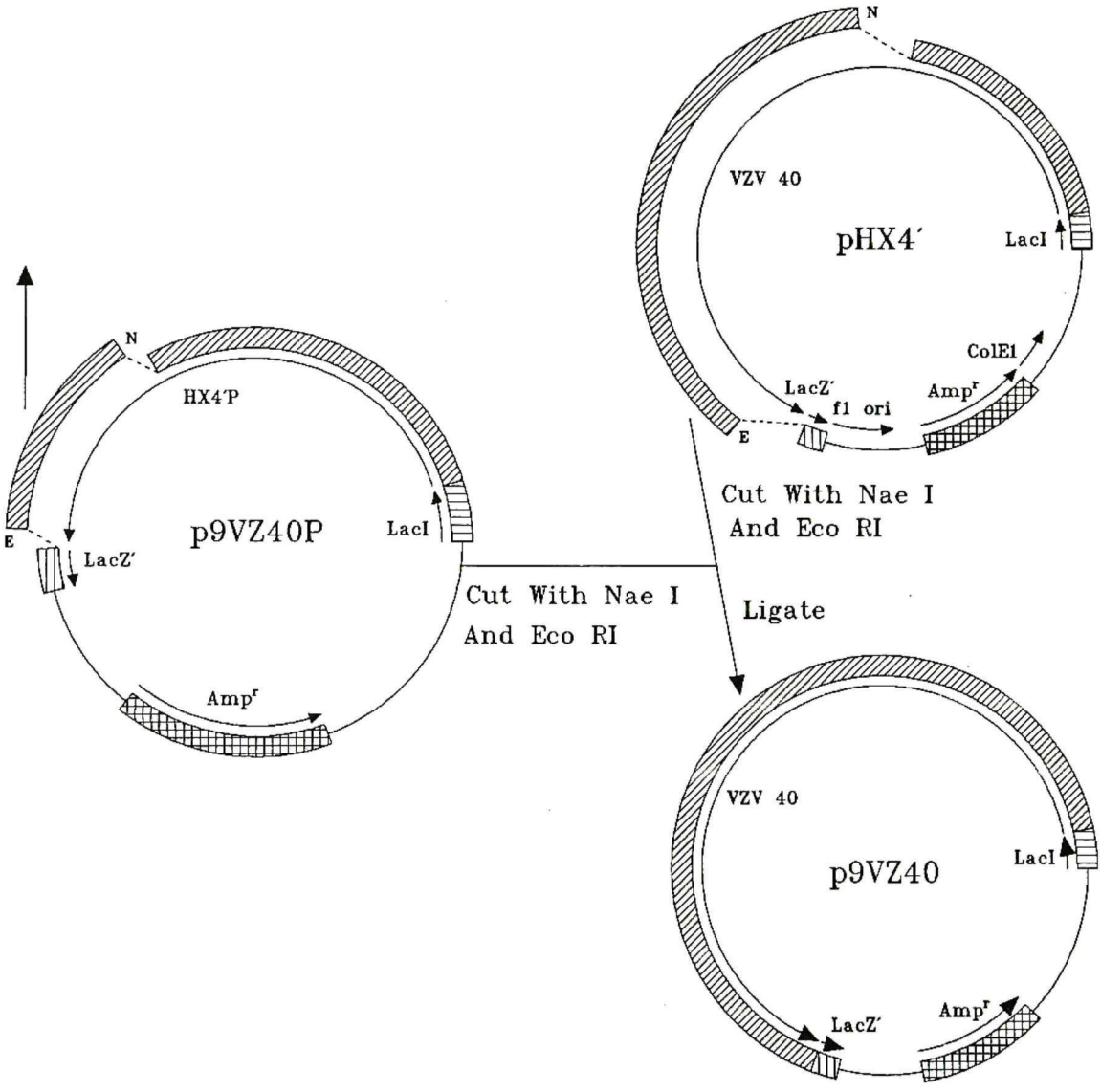

N
VZV 40
pHX4′
LacI
ColE1
LacZ′
f1 ori
Amp^r
E
Cut With Nae I
And Eco RI
Ligate
N
HX4′P
p9VZ40P
LacI
E
LacZ′
Amp^r
Cut With Nae I
And Eco RI
VZV 40
p9VZ40
LacI
LacZ′
Amp^r

Figure 66. The cloning of pVZ40SC. The 5,110 bp Hind III/Eco RI fragment of p9VZ40 was inserted into the pSC11 Sma I site to give pVZ40SC.

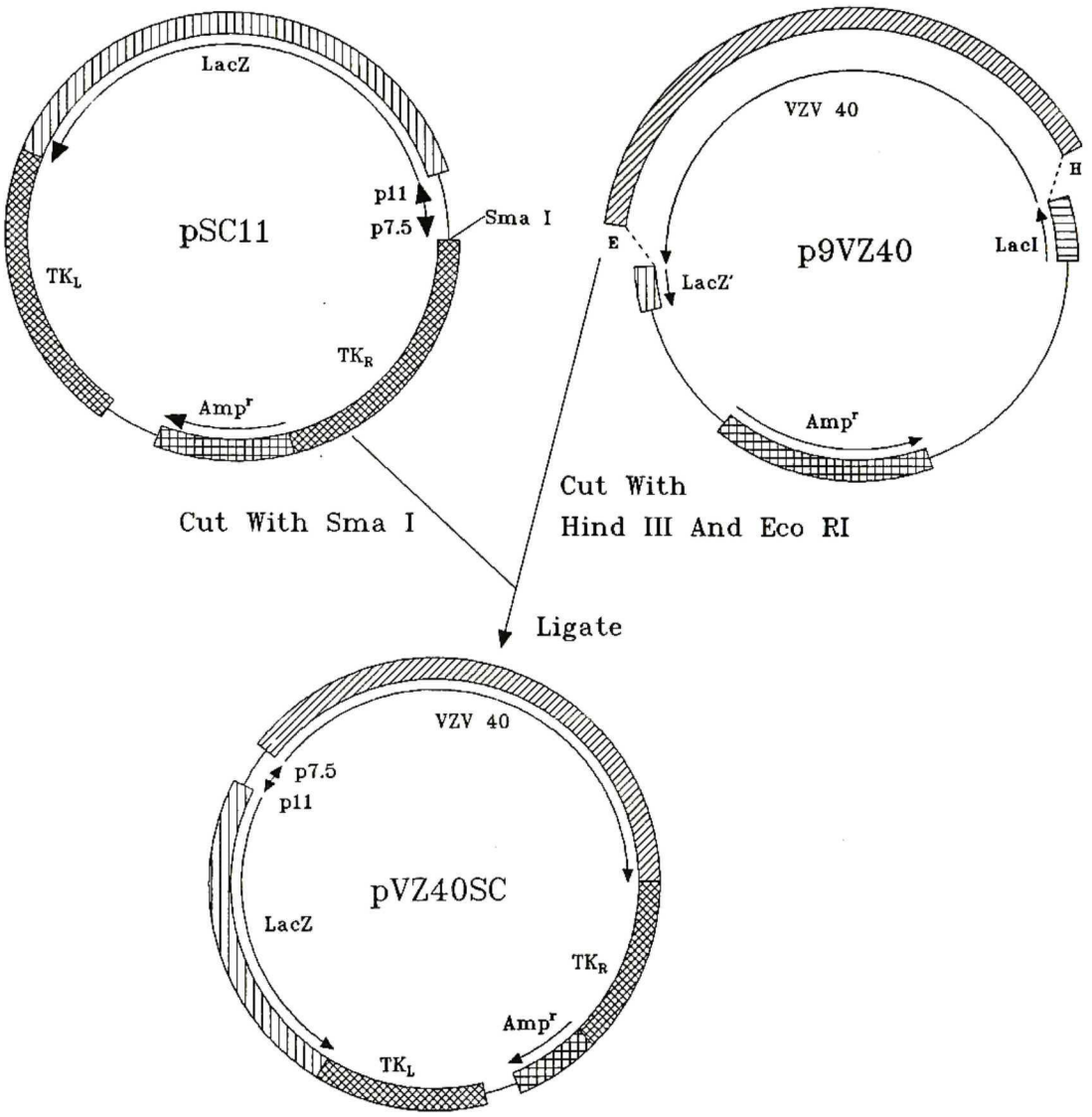

LacZ
p11
p7.5
pSC11
Sma I
TK$_L$
TK$_R$
Amp^r
Cut With Sma I
VZV 40
H
E
LacZ'
p9VZ40
LacI
Amp^r
Cut With
Hind III And Eco RI
Ligate
VZV 40
p7.5
p11
pVZ40SC
LacZ
TK$_R$
Amp^r
TK$_L$

Figure 67. The cloning of p9VZ47. The 1,867 bp Hga I fragment of the VZV BamG clone was inserted into the Sma I site of pUC9 to give p9VZ47.

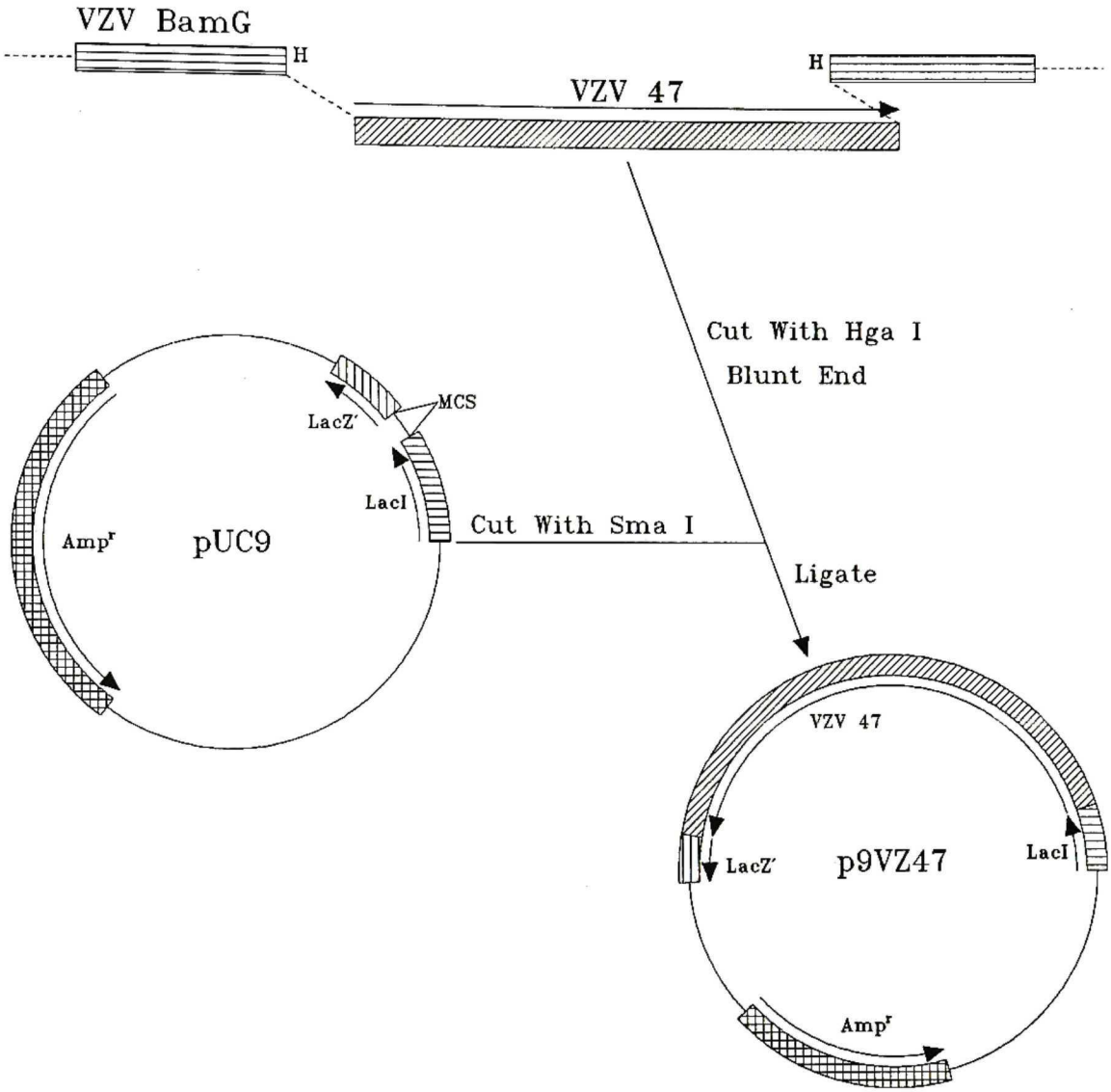

VZV BamG
H
H
VZV 47
Cut With Hga I
Blunt End
MCS
LacZ'
LacI
Amp^r
pUC9
Cut With Sma I
Ligate
VZV 47
LacZ'
p9VZ47
LacI
Amp^r

Figure 68. The cloning of pVZ47SC. The 1,867 bp Hga I fragment of the VZV BamG clone was inserted into the Sma I site of pSC11 to give pVZ47SC.

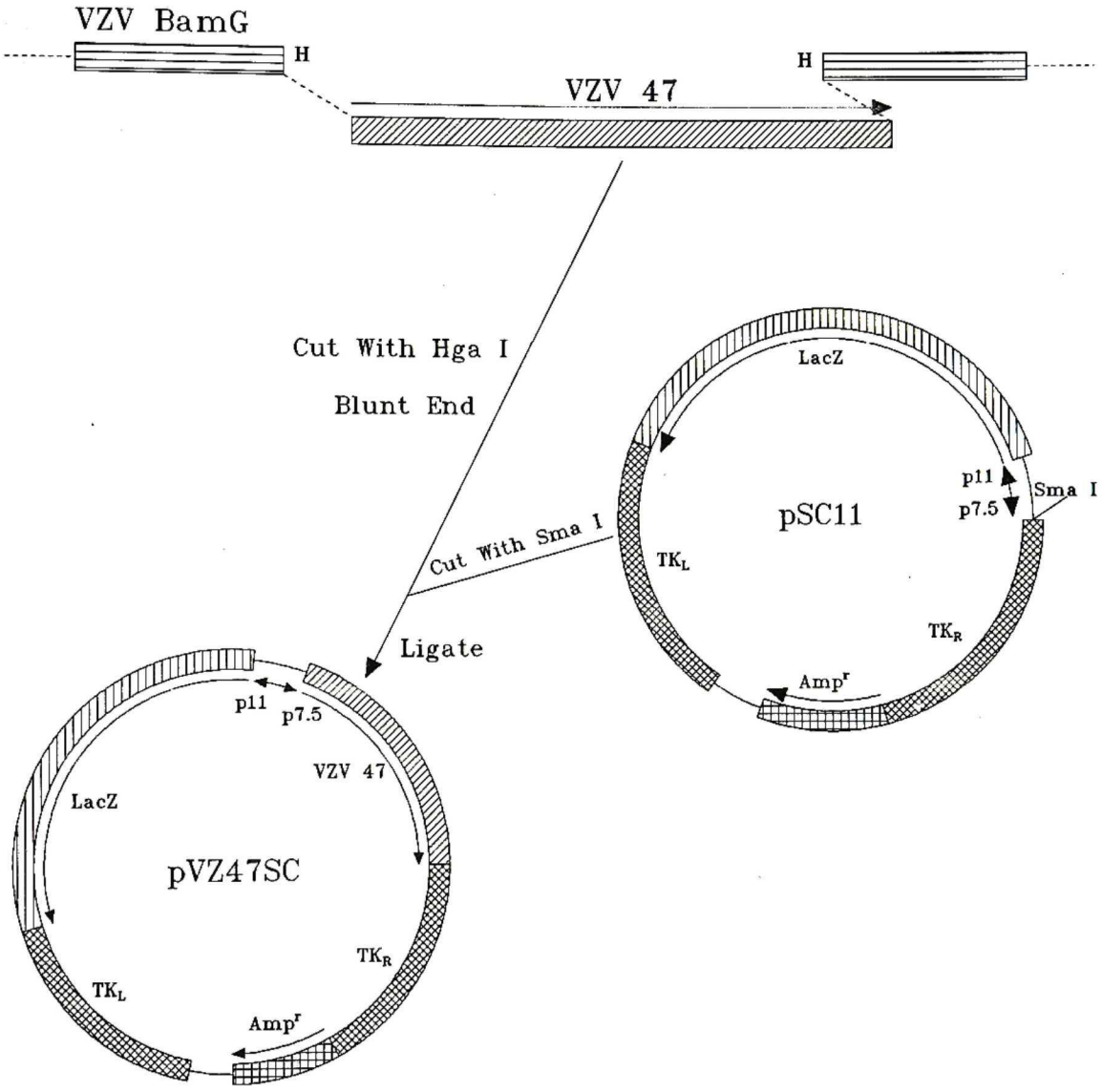

VZV BamG
H
H
VZV 47
Cut With Hga I
Blunt End
Cut With Sma I
Ligate
LacZ
p11
p7.5
Sma I
pSC11
TK_L
TK_R
Amp^r
p11
p7.5
VZV 47
LacZ
pVZ47SC
TK_R
TK_L
Amp^r

Figure 69. The cloning of pVZ47BS. The 1,875 bp Bam HI/Mst II fragment of pVZ47SC was inserted into the Sma I site of pBS to give pVZ47BS.

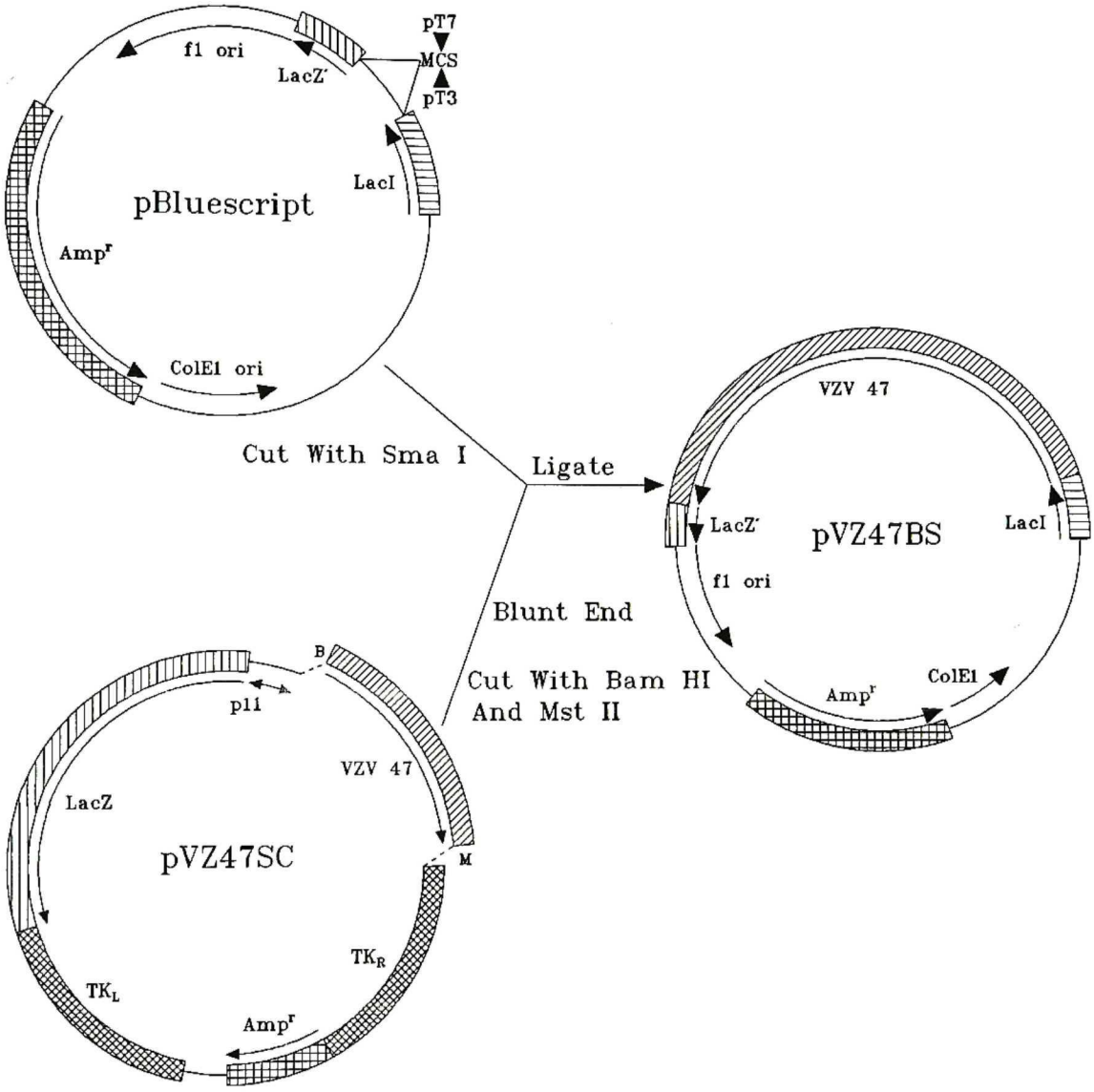

pT7
MCS
pT3
f1 ori
LacZ'
LacI
pBluescript
Amp^r
ColE1 ori
Cut With Sma I
Ligate
VZV 47
LacZ'
pVZ47BS
LacI
f1 ori
Amp^r
ColE1
Blunt End
Cut With Bam HI
And Mst II
B
p11
VZV 47
LacZ
pVZ47SC
M
TK_R
TK_L
Amp^r

Figure 70. The cloning of pVZ66ABS. The 974 bp Acc I fragment of the VZV EcoA clone was inserted into the pBS Acc I site to give pVZ66ABS.

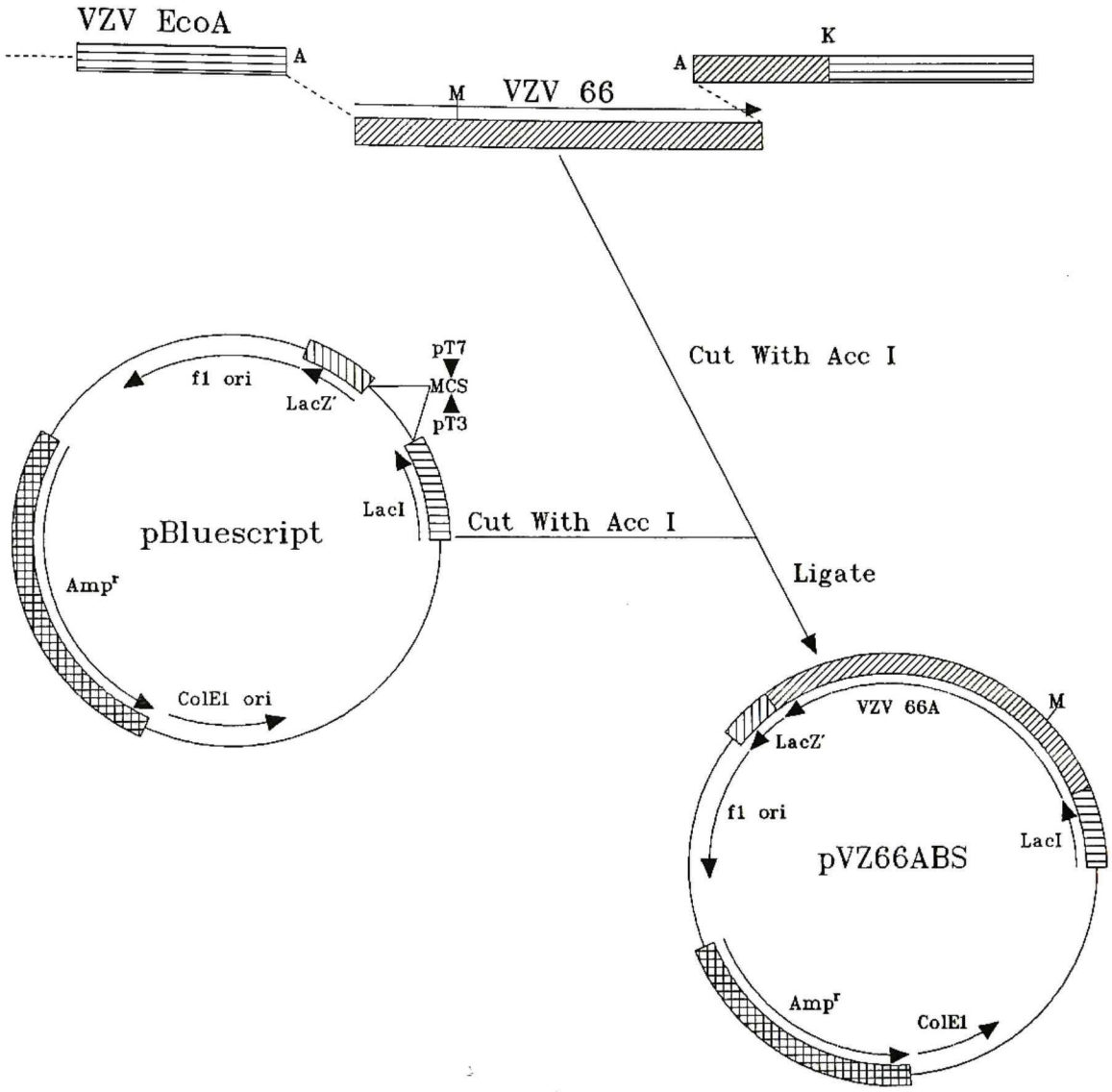

VZV EcoA
A
K
A
M
VZV 66
Cut With Acc I
pT7
MCS
pT3
f1 ori
LacZ'
LacI
pBluescript
Amp^r
ColE1 ori
Cut With Acc I
Ligate
VZV 66A
M
LacZ'
f1 ori
LacI
pVZ66ABS
Amp^r
ColE1

Figure 71. The cloning of pVZ66BS. The 1,839 bp Mlu I/Kpn I fragment of VZV clone EcoA was inserted into pVZ66ABS cut with Mlu I and Kpn I to give pVZ66BS.

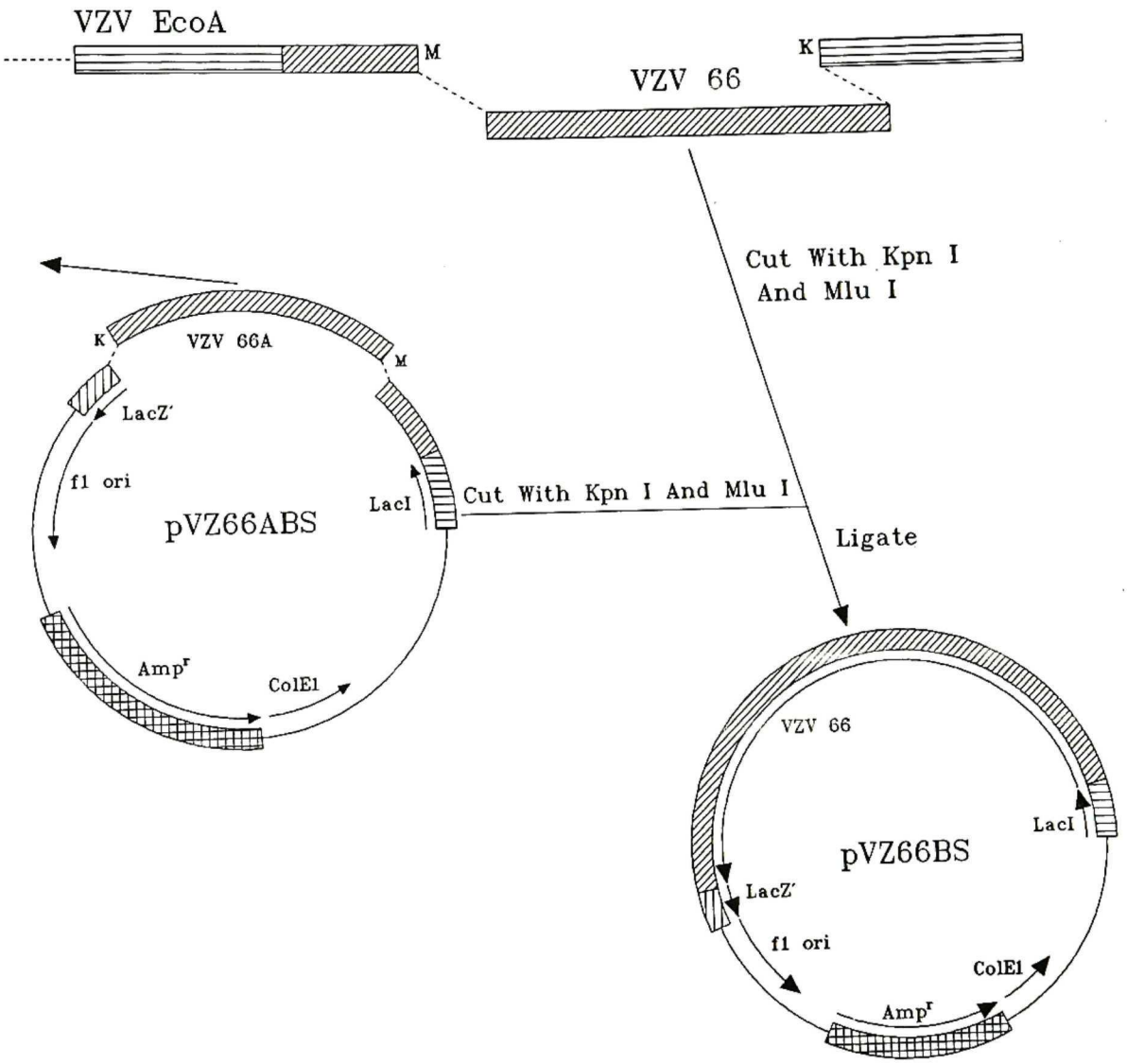
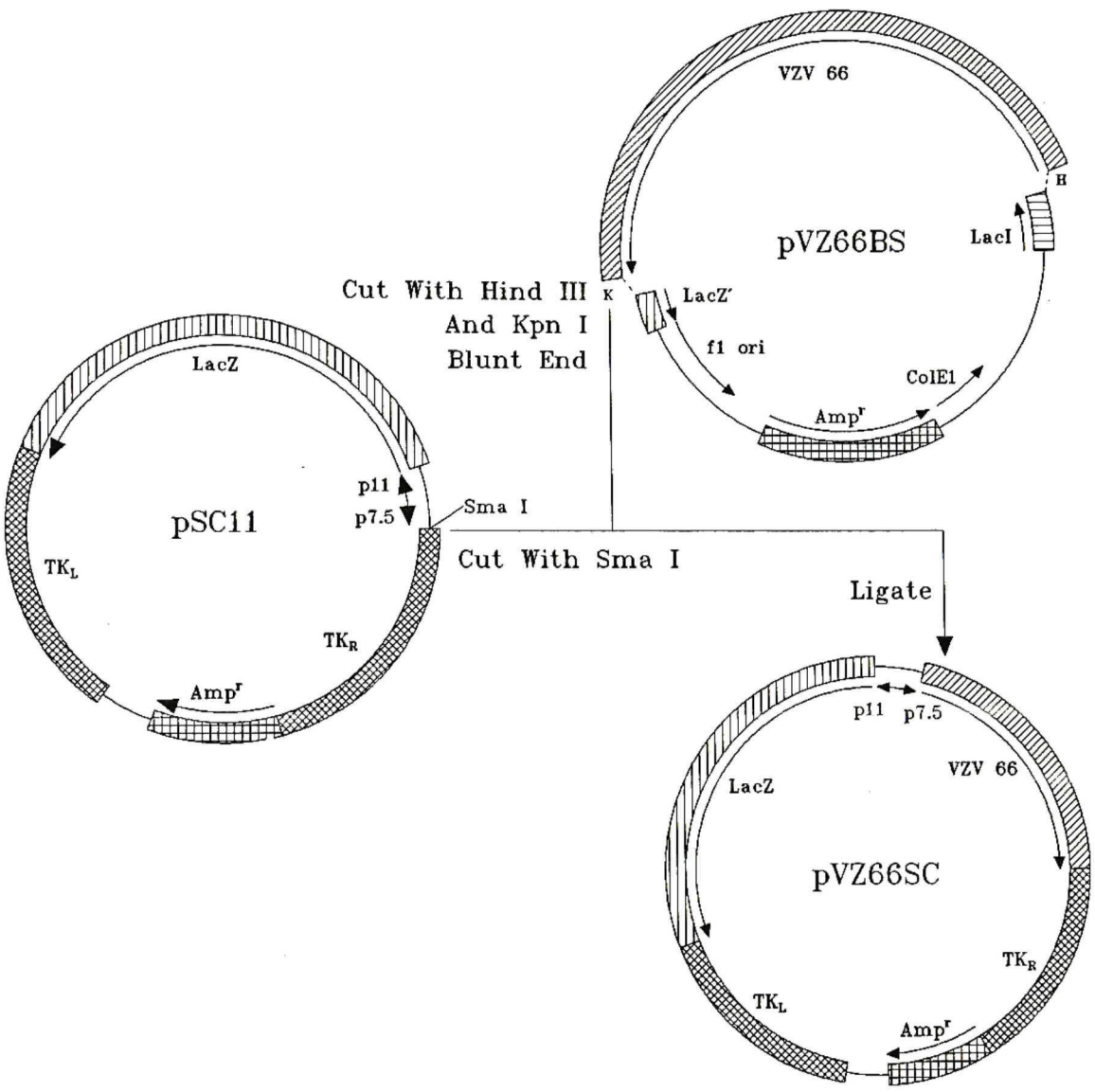

VZV EcoA
M
K
VZV 66
Cut With Kpn I
And Mlu I
K
VZV 66A
M
LacZ'
f1 ori
pVZ66ABS
LacI
Cut With Kpn I And Mlu I
Ligate
Amp^r
ColE1
VZV 66
LacI
pVZ66BS
LacZ'
f1 ori
ColE1
Amp^r

Figure 72. The cloning of pVZ66SC. The 2,110 bp Hind III/Kpn I fragment of pVZ66BS was inserted into the Sma I site of pSC11 to give pVZ66SC.


VZV 66
H
pVZ66BS
LacI
Cut With Hind III
And Kpn I
Blunt End
K
LacZ'
f1 ori
ColE1
Amp^r
LacZ
p11
p7.5
pSC11
Sma I
TK$_L$
TK$_R$
Amp^r
Cut With Sma I
Ligate
p11
p7.5
VZV 66
LacZ
pVZ66SC
TK$_R$
TK$_L$
Amp^r

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